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(54) Title: <b>C-C CHEMOKINE RECEPTOR 3: CKP-3 OR Eos-L2</b>			
(57) Abstract			
<p>The present invention relates to isolated and/or recombinant nucleic acids which encode a mammalian (e.g., human) receptor protein designated C-C Chemokine Receptor 3 (CKR-3) or Eos L2, and to proteins or polypeptides, referred to herein as isolated, recombinant mammalian CKR-3 receptors. The invention further relates to recombinant nucleic acid constructs comprising a nucleic acid which encodes a receptor protein of the present invention or a portion thereof; to host cells comprising such constructs, useful for the production of recombinant CKR-3 receptors or polypeptides; and to antibodies reactive with the receptors, which are useful in research and diagnostic applications. Also provided are methods of use of the nucleic acids, proteins, and host cells to identify ligands, inhibitors (e.g., antagonists) or promoters (agonists) of receptor function. Administration of a compound which inhibits or promotes receptor function to an individual in need of therapy provides a new approach to selective modulation of leukocyte function, which is useful in a variety of inflammatory and autoimmune diseases, or in the treatment of infections. As a major leukocyte chemokine receptor present in leukocytes such as eosinophils and lymphocytes, the receptor provides a key target for drug screening and design.</p>			

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## C-C Chemokine Receptor 3: CKR or Eos-L2

DescriptionRelated Applications

- This application is a continuation-in-part of U.S. Serial No. 08/375,199, filed January 19, 1995, the teachings of which are incorporated herein by reference in their entirety.

Government Support

- Work described herein was supported in whole or in part by a U.S. government grant. The U.S. government has certain rights in this invention.

Background

- Chemokines, also referred to as intecrines, are soluble, low molecular weight members of the cytokine family which have chemoattractant function. Chemokines are capable of selectively inducing chemotaxis of the formed elements of the blood (other than red blood cells), including leukocytes such as monocytes, macrophages, eosinophils, basophils, mast cells, and lymphocytes, such as T cells, B cells, and polymorphonuclear leukocytes (neutrophils)). In addition to stimulating chemotaxis, other changes can be selectively induced by chemokines in responsive cells, including changes in cell shape, transient rises in the concentration of intracellular free calcium ( $[Ca^{2+}]_i$ ), granule exocytosis, integrin upregulation, formation of bioactive lipids (e.g., leukotrienes) and respiratory burst, associated with leukocyte activation. Thus, the chemokines are early triggers of the inflammatory response, causing inflammatory

mediator release, chemotaxis and extravasation to sites of infection or inflammation.

The chemokines characterized to date are related in primary structure. They share four conserved cysteines, which form disulphide bonds. cDNA cloning and biochemical characterization of several chemokines has revealed that the proteins have a leader sequence of 20-25 amino acids, which is cleaved upon secretion to yield a mature protein of approximately 92-99 amino acids. Based on the conserved cysteine motif, the family is divided into two branches, designated as the C-C chemokines ( $\beta$  chemokines) and the C-X-C chemokines ( $\alpha$  chemokines), in which the first two conserved cysteines are adjacent or are separated by an intervening residue, respectively. Baggiolini, M. and C.A. Dahinden, *Immunology Today*, 15: 127-133 (1994)).

The C-X-C chemokines include a number of chemoattractants which are potent chemoattractants and activators of neutrophils, such as interleukin 8 (IL-8), PF4 and neutrophil-activating peptide 2 (NAP-2). The C-C chemokines include molecules such as human monocyte chemotactic proteins 1-3 (MCP-1, MCP-2 and MCP-3), RANTES (Regulated on Activation, Normal T Expressed and Secreted), and the macrophage inflammatory proteins 1 $\alpha$  and 1 $\beta$  (MIP-1 $\alpha$  and MIP-1 $\beta$ ), which have been characterized as chemoattractants and activators of monocytes or lymphocytes, but do not appear to be chemoattractants for neutrophils. For example, recombinant RANTES is a chemoattractant for monocytes, as well as for memory T cells in vitro (Schall, T.J. et al., *Nature*, 347: 669-671 (1990)). More recently a chemokine called lymphotactin with a single cysteine pair in the molecule has been identified which attracts lymphocytes (Kelner, G.S., et al., *Science*, 266: 1395-1359 (1994)).

The C-C chemokines are of great interest because of their potential role in allergic inflammation. For

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example, MCP-1 induces exocytosis of human basophils, resulting in release of high levels of inflammatory mediators, such as histamine and leukotriene C<sub>4</sub>. Similarly, there is great interest in the receptors for the C-C chemokines, which trigger these cellular events in response to chemokine binding. A receptor for C-C chemokines has recently been cloned and is reported to bind MIP-1 $\alpha$  and RANTES. Accordingly, this MIP-1 $\alpha$ /RANTES receptor was designated C-C chemokine receptor 1 (CKR-1; Neote, K. et al., *Cell*, 72: 415-425 (1993); Horuk, R. et al., WO 94/11504, published May 26, 1994; Gao, J.-I. et al., *J. Exp. Med.*, 177: 1421-1427 (1993)). An MCP-1 receptor has also been cloned (Charo, I.F. et al., *Proc. Natl. Acad. Sci. USA*, 91: 2752 (1994)). This receptor, designated CKR-2, is reported to bind MCP-1 with high affinity and MCP-3 with lower affinity (Charo, I.F., et al., *Proc. Natl. Acad. Sci. USA*, 91: 2752-2756 (1994)). CKR-2 has been shown to exist in two isoforms resulting from the use of an alternative splice site in isoform A producing a distinct cytoplasmic tail. Isoform B, which is not spliced in this region, has been shown to be a functional receptor for MCP-1 and MCP-3 in binding and signal transduction assays (Charo, I.F., et al., *Proc. Natl. Acad. Sci. USA*, 91: 2752-2756 (1994); Myers, S.J., et al., *J. Biol. Chem.*, 270: 5786-5792 (1995)). More recently, a new receptor called CKR-4 has been described; cRNA from this receptor was reported to produce a Ca<sup>2+</sup> activated chloride current in response to MCP-1, MIP-1 $\alpha$ , and RANTES when injected in to *X. laevis* oocytes (Power, C.A., et al., *J. Biol. Chem.*, 270: 19495-19500 (1995)).

The MCP-1 receptor (CKR-2) and C-C chemokine receptor 1 are predicted to belong to a superfamily of seven transmembrane spanning G-protein coupled receptors (Gerard C., and Gerard, N.P., *Annu. Rev. Immunol.*, 12: 775-808

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(1994); Gerard C., and Gerard N.P., *Curr. Opin. Immunol.*, 6: 140-145 (1994)). This family of G-protein coupled (serpentine) receptors comprises a large group of integral membrane proteins, containing seven transmembrane-spanning regions. The ligands of these receptors include a diverse group of molecules, including small biogenic amine molecules, such as epinephrine and norepinephrine, peptides, such as substance P and neurokinins, and larger proteins, such as chemokines. The receptors are coupled to G proteins, which are heterotrimeric regulatory proteins capable of binding GTP and mediating signal transduction from coupled receptors, for example, by the production of intracellular mediators.

The cloning and sequencing of two IL-8 receptor cDNAs reveals that these C-X-C receptor proteins also share sequence similarity with seven transmembrane-spanning G protein-coupled receptor proteins (Murphy P.M. and H.L. Tiffany, *Science*, 253: 1280-1283 (1991); Murphy et al., WO 93/06299; Holmes, W.E. et al., *Science*, 253: 1278-1280 (1991)). Additional receptors for chemotactic proteins such as anaphylatoxin C5a and bacterial formylated tripeptide fMLP have been characterized by cloning and been found to encode receptor proteins which also share sequence similarity to these seven transmembrane-spanning proteins (Gerard, N.P. and C. Gerard, *Nature*, 349: 614-617 (1991); Boulay, F. et al., *Biochemistry*, 29: 11123-11133 (1990)). Although a number of other proteins with significant sequence similarity and similar tissue and leukocyte subpopulation distribution to known chemokine receptors have been identified and cloned, the ligands for these receptors remain undefined. Thus, these proteins are referred to as orphan receptors.

The isolation and characterization of additional genes and the encoded receptors, and the characterization of the corresponding ligands, is essential to an understanding of

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the interaction of chemokines with their target cells and the events stimulated by this interaction, including chemotaxis and cellular activation of leukocytes.

#### Summary of the Invention

5       The present invention relates to isolated and/or recombinant nucleic acids which encode a mammalian (e.g., human) receptor protein designated C-C Chemokine Receptor 3 (CKR-3). The invention further relates to recombinant  
10       nucleic acid constructs, such as plasmids or retroviral vectors, which contain a nucleic acid which encodes a receptor protein of the present invention, or portions of said receptor. The nucleic acids and constructs can be used to produce recombinant receptor proteins. In another  
15       embodiment, the nucleic acid encodes an antisense nucleic acid which can hybridize with a second nucleic acid encoding a receptor of the present invention, and which, when introduced into cells, can inhibit the expression of receptor.

      Another aspect of the present invention relates to  
20       proteins or polypeptides, referred to herein as isolated, recombinant mammalian CKR-3 receptors. The recombinant CKR-3 receptors or polypeptides can be produced in host cells as described herein. In one embodiment, a receptor protein is characterized by high affinity binding of one or  
25       more chemokines, such as eotaxin, RANTES and/or MCP-3, and/or the ability to stimulate a (one or more) cellular response(s) (e.g., chemotaxis, exocytosis, release of one or more inflammatory mediators).

      Antibodies reactive with the receptors can be produced  
30       using the receptors or portions thereof as immunogen or cells expressing receptor protein or polypeptide, for example. Such antibodies or fragments thereof are useful in therapeutic, diagnostic and research applications, including the purification and study of the receptor



proteins, identification of cells expressing surface receptor, and sorting or counting of cells.

Also encompassed by the present invention are methods of identifying ligands of the receptor, as well as  
5 inhibitors (e.g., antagonists) or promoters (agonists) of receptor function. In one embodiment, suitable host cells which have been engineered to express a receptor protein or polypeptide encoded by a nucleic acid introduced into said cells are used in an assay to identify and assess the  
10 efficacy of ligands, inhibitors or promoters of receptor function. Such cells are also useful in assessing the function of the expressed receptor protein or polypeptide.

According to the present invention, ligands, inhibitors and promoters of receptor function can be  
15 identified and further assessed for therapeutic effect. Ligands and promoters can be used to stimulate normal receptor function where needed, while inhibitors of receptor function can be used to reduce or prevent receptor activity. Thus, the present invention provides a new  
20 strategy of anti-inflammatory therapy, useful in a variety of inflammatory and autoimmune diseases, comprising administering an inhibitor of receptor function to an individual (e.g., a mammal). In contrast, stimulation of receptor function by administration of a ligand or promoter  
25 to an individual provides a new approach to selective stimulation of leukocyte function, which is useful, for example, in the treatment of parasitic infections.

#### Brief Description of the Drawings

Figure 1A-1C illustrates the nucleotide sequence  
30 determined from a genomic clone encoding a human CKR-3 protein also referred to as Eos L2 receptor (SEQ ID NO:1), and the predicted amino acid sequence of the protein encoded by the open-reading frame (SEQ ID NO:2).

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Figure 2A-2B illustrates the nucleotide sequence determined from the cDNAs encoding a human CKR-3 receptor (SEQ ID NO:3), and the predicted amino acid sequence of the protein encoded by the open-reading frame (SEQ ID NO:4).

5        Figure 3 is an illustration of one type of transendothelial chemotaxis assay. A culture insert is placed into a container, such as a well in a 24-well plate, creating a first and second chamber within the well. ECV304 endothelial cells are grown in a monolayer on the  
10       polycarbonate membrane on the inner side of the insert. Cells to be assessed for a response to a substance (e.g., a chemokine) are introduced into the top chamber and the substance is introduced into the bottom chamber. Chemotaxis can be assessed by detecting cells which migrate  
15       through the endothelial layer into the bottom chamber, by removing the insert and detecting or counting cells by a suitable method. For example, cells in the bottom chamber can be collected and assessed by flow cytometry (e.g., FACS analysis, light scattering).

20       Figure 4 is a histogram illustrating the chemotaxis of human eosinophils in response to various chemokines. Human eosinophils were purified using a standard protocol, and assessed by microscopy for their response to various chemokines in a 24 well transendothelial chemotaxis assay  
25       (cells per high power field (HPF)).

Figures 5A-5B are histograms illustrating the response of HL-60 cells (Figure 5A) and butyrate-differentiated HL-60 cells (Figure 5B) to various chemokines. Chemotaxis assays were performed using endothelial coated inserts, and  
30       lasted 1.5 hours. Results are representative of 10 different experiments.

Figure 6 is an illustration of a FACS analysis of various clones of L1-2 pre-B cells transfected with Eos L2. Cells from over 200 clones were stained with M2 anti-FLAG  
35       Mab followed by anti-mouse Ig-FITC. (Y-axis, number of

cells; X-axis, fluorescence). In the negative control (PAUL 001), transfected cells were stained with an irrelevant antibody.

Figure 7 is a histogram illustrating the binding of RANTES and MIP-1 $\alpha$  to human eosinophils. Purified normal human eosinophils were incubated with 0.1 nM  $^{125}$ I-labeled MIP-1 $\alpha$  or RANTES ("Hot") in the presence or absence of various cold chemokines (MIP-1 $\alpha$ , RANTES, IL-8, MCP-1, MCP-3) at 250 nM.

Figure 8 is a graph illustrating inhibition of the binding of  $^{125}$ I-labeled RANTES to human eosinophils by various cold chemokines (RANTES, MIP-1 $\alpha$ , MCP-1 and MCP-3). Human eosinophils were incubated with 0.1 nM radiolabeled RANTES and the indicated concentrations of cold chemokines. The data plotted are the means and standard deviations of duplicates for each sample.

Figure 9 is a histogram illustrating the binding of 0.1 nM radiolabeled MIP-1 $\alpha$  or RANTES ("Hot") to butyric acid differentiated HL-60 cells in the absence or presence of cold chemokines (250 nM).

Figure 10 is a histogram illustrating the binding of 0.1 nM  $^{125}$ I-labeled ("Hot") RANTES or 0.1 nM  $^{125}$ I-labeled ("Hot") MCP-3 to Eos L2 infected SF9 cells (cpm, counts per minute). (From left to right: Hot Rantes only; Hot Rantes + Cold Rantes; Hot MCP-3 only; Hot MCP-3 + cold MCP-3).

Figures 11A-11B are graphs illustrating the binding of MCP-3 to butyrate-differentiated HL-60 cells. In Figure 11A, binding of 0.1 nM radiolabeled MCP-3 was assessed in the presence of various concentrations of cold MCP-3. Figure 11B is a Scatchard plot calculated from data in Figure 11A.

Figures 12A-12B are an illustration of the results of a FACS analysis of human eosinophils (Figure 12A) and lymphocytes (Figure 12B) for expression of Eos L2, using

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monoclonal antibody (Mab) LS26-5H12 as first antibody, and FITC-anti-mouse Ig as second antibody. "Neg" indicates a negative control in which non-specific antibody was used as first antibody.

5        Figures 13A-13D are graphs illustrating CKR-3 expression on leukocytes as determined using Mab LS26-5H12 and flow cytometry. Leukocyte subsets were stained with anti-CKR-3 Mab LS26-5H12 (solid lines) or an IgG<sub>1</sub> isotype-matched control antibody (MOPC-21) (shaded profile). Figure 13A, eosinophils; Figure 13B, T Cells; 10        Figure 13C, monocytes; Figure 13D, neutrophils. Dead cells were excluded based on propidium iodide staining.

Figures 14A-14C are graphs illustrating cell surface staining of L1.2 cells transiently transfected with a CKR-3 15        receptor (Figure 14A), mock-transfected L1.2 control cells (Figure 14B), or cell line E5 (a stable L1.2 CKR-3 transfectant) (Figure 14C) with an anti-CKR-3 monoclonal antibody (LS26-5H12, solid line). Background staining with control monoclonal antibody MOPC-21 is also shown (shaded 20        profile).

Figures 15A-15D are graphs illustrating the results of competitive ligand binding of radiolabeled human eotaxin to the E5 cell line (a stable L1-2 cell line transfected with a CKR-3 receptor; Figure 15A) or to human eosinophils 25        (Figure 15B). Cells were incubated with 0.6 nM <sup>125</sup>I-labeled eotaxin and various concentrations of unlabeled eotaxin (O), RANTES (Δ), or MCP-3 (□). After 60 minutes at room temperature, cell pellets were washed and counted. Scatchard plots of unlabeled eotaxin competition were 30        calculated from the data (Figure 15C, E5 cell line; Figure 15D, eosinophils).

Figure 16 is a histogram illustrating the inhibition by various chemokines of human eotaxin binding to the E5 cell line. E5 cells (stable L1-2/CKR-3 transfectants) were

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incubated with 0.6 nM radiolabeled eotaxin and 250 nM unlabeled chemokines or no competitor as indicated.

Figures 17A-17C are histograms illustrating chemotaxis of L1.2 cells and L1.2 receptor transfectants.  $1 \times 10^6$  cells of the E5 cell line (stable L1-2/CKR-3 transfectants) (Figure 17A), the parental L1.2 cell line (Figure 17B), or an IL-8 RB L1.2 receptor transfectant line LSLW-2 (Figure 17C) were placed in the top chamber and chemokines placed in the bottom chamber at the concentrations specified. Migration was allowed for 4 hours and cells migrating to the bottom chamber were counted. All assays were performed in duplicate and the results representative of at least three separate experiments. Chemokines are listed along the x-axis, number of cells migrated along the y-axis, and concentration of chemokine along the z-axis.

Figures 18A-18B are graphs illustrating the chemotactic response of eosinophils from two different individuals. The response resembles that of CKR-3 L1.2 transfectants. Donor to donor variation of chemotactic responses of eosinophils to eotaxin, RANTES, MCP-3, and MIP-1 $\alpha$  was observed. Eosinophils were purified from blood, and assessed for their chemotactic response to various concentrations of chemokines. Values are from a representative experiment of at least 4 performed, using the same two blood donors.

Figure 19 is a graph illustrating the binding of  $^{125}\text{I}$ -labeled RANTES to a membranes from a stable cell line (A31-293-20) obtained by transfecting 293 cells with the A31 cDNA clone (square with central dot) as compared with binding to membranes from untransfected 293 cells (filled circles).

Figure 20 is a histogram illustrating the binding of  $^{125}\text{I}$ -labeled MCP-3 to a membranes from a stable cell line (A31-293-20) obtained by transfecting 293 cells with the

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A31 cDNA clone as compared with binding to membranes from untransfected 293 cells. Binding of labeled MCP-3 to membranes from transfected (A31-20) or untransfected (UT293) cells was determined in the absence of cold MCP-3 (0 nM) or in the presence of cold MCP-3 (100 nM).

Figure 21 is a histogram illustrating the specificity of binding, which was assessed by determining the amount of bound <sup>125</sup>I-labeled MCP-3 which could be displaced by cold MCP-3 from membranes of transfected (A31-20) or untransfected (UT293) cells.

#### Detailed Description of the Invention

As described herein, nucleic acids encoding a novel human receptor, designated Eos L2 or C-C chemokine receptor 3 (CKR-3) have been isolated. Both human genomic and cDNA clones have been characterized. The cDNA clone was isolated from an eosinophil cDNA library constructed from eosinophils obtained from a patient with hypereosinophilic syndrome. Sequence analysis of the clones revealed a gene containing an open reading frame of 1065 nucleotides encoding a predicted protein of 355 amino acids (Figures 1A-1C and 2A-2B; SEQ ID NOS: 2 and 4), which shares amino acid sequence similarity with other C-C chemokine receptors, which are believed to be G protein-coupled receptors and to have a similar structure of seven transmembrane spanning regions.

The predicted proteins encoded by CKR-3 genomic and cDNA clones contain four cysteine residues, one in each of the extracellular domains at positions 24, 106, 183 and 273 (SEQ ID NOS:2 and 4). Cysteines at these positions are conserved in all chemokine receptors, including CKR-1, CKR-2, CKR-4, IL8-RA and IL8-RB. In addition, this receptor contains an amino acid motif, DRYLAIVHA (residues 130-138) (SEQ ID NOS: 2 and 4), which is also highly

conserved among C-X-C and C-C chemokine receptors and is predicted to be intracellular. There are two consensus sites for protein kinase C phosphorylation (Kishimoto, A., et al., *J. Biol. Chem.*, 260: 12492-12499 (1985); Woodgett, J.R., *Eur. J. Biochem.*, 161: 177-184 (1986)), one in the third intracellular loop at AA position 231, and one in the cytoplasmic tail at AA position 333. In addition, there are eight serine/threonine residues in the cytoplasmic tail, which may serve as phosphorylation sites for G-protein coupled receptor kinases such as those isolated from neutrophils (Haribabu, B. and R. Snyderman, *Proc. Natl. Acad. Sci. USA*, 90: 9398 (1993)) or other related family members (Benovic, J.L., and Gomez, J., *J. Biol. Chem.*, 268: 19521-19527 (1993); Kunapuli, P., and Benovic, J.L., *Proc. Natl. Acad. Sci. USA*, 90: 5588-5594 (1993)). Serine/threonine rich cytoplasmic tails are also a common feature of chemokine receptors. Unlike CKR-1, CKR-2, CKR-4, IL-8RA and IL-8RB receptors, CKR-3 does not contain sites for N-linked glycosylation in any extracellular domain. The CKR-3 receptor protein is distinct from C-C chemokine receptor 1, also referred to as the MIP-1 $\alpha$ /RANTES receptor.

The nucleic acid sequences obtained from genomic and cDNA libraries were co-linear, with the following exceptions. Upstream of the initiation codon the two sequences diverge (at position 78 of Figure 2A). The genomic clone appears to have an intron which separates the promoter and most of the 5' untranslated region from the coding region. This genomic arrangement is similar to that found in other seven transmembrane-spanning chemoattractant receptors (Gerard, N.P., et al., *Biochemistry*, 32: 1243-1250 (1993); Murphy, P.M., et al., *Gene*, 133: 285-290 (1993)) including IL-8 RA and RB (Ahuja, S.K., et al., *J. Biol. Chem.*, 269: 26381-89 (1994); Sprenger, H., et al., *J. Biol. Chem.*, 269: 11065-11072 (1994); Sprenger, H., et al.,

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*J. Immunol.*, 153: 2524-2532 (1994)) and CKR-1 (Gao, J.L., et al., *J. Exp. Med.*, 177: 1421-1427 (1993)). Furthermore, examination of the genomic sequence around the point of divergence reveals a canonical splice acceptor sequence.

- 5 Initial sequence information revealed two regions in which the cDNA sequence appeared to be shifted in frame, resulting from an insertion of a base followed by the deletion of a base, or the deletion of a base followed by the insertion of a base. These alterations resulted in
- 10 four contiguous amino acid differences in the predicted proteins at positions 263-266 and 276-279, respectively. Other differences led to amino acid differences at positions 182, 196, 197, and 315 of the predicted proteins. The nucleotide sequence presented in SEQ ID NO:5 is a
- 15 consensus sequence, which includes regions which were sequenced in both clones, and was constructed by simple alignment (base for base) of the initial nucleic acid sequences. SEQ ID NO:6, in which the initial amino acid differences between the cDNA and genomic clones are
- 20 indicated by Xaa, represents the predicted protein of SEQ ID NO:5. However, further sequence analysis revealed that nucleotide sequences of the open reading frames appear to differ only at a position corresponding to nucleotides 918-919 of Figure 2B. The genomic clone has a CG at this
- 25 position, while the cDNA clone has a GC at this position. Thus, the genomic clone codes for threonine (ACG) at position 276 and the cDNA clone codes for serine (AGC) at position 276. The difference may be due to a sequencing ambiguity, or an error introduced into the cDNA during
- 30 reverse transcription. Alternatively, the conservative substitution (serine/threonine) could be due to polymorphism between individuals. Another alternative is that the differences are due to mutation of the receptor gene in the eosinophils of the patient from which RNA for cDNA library
- 35 construction was obtained.



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Monoclonal and polyclonal antibodies specific for a C-C chemokine receptor 3 of human origin were produced using an N-terminal synthetic peptide of the receptor. FACS (fluorescence activated cell sorting) analysis using one of the monoclonal antibodies revealed significant expression of this receptor on human eosinophils, but not on leukocytes including monocytes, neutrophils, lymphocytes, T cells, T cell blasts (produced by activation with CD3 MAb) (Figures 13A-13D). This pattern of expression was confirmed by Northern analysis with RNA from highly purified leukocyte subsets. However, in some experiments, CKR-3 mRNA or receptor was detected in T lymphocytes; accordingly, it is possible that CKR-3 is expressed on a subset of T lymphocytes (Example 5).

Genomic and cDNA clones were also expressed in a variety of systems. Antibody was used to detect expression of receptor from the genomic clone on transfected mammalian cells and baculovirus-transfected insect cells. Stable transfectants of mammalian cells expressing CKR-3 were constructed, and the encoded receptor was shown to bind radiolabeled eotaxin specifically and with high affinity, comparable to the binding affinity observed with eosinophils. Studies with transfected mammalian cells indicated that the receptor also binds RANTES and MCP-3 specifically and with high affinity, but not other CC or CXC chemokines tested. Consistent with the binding data, as shown herein, receptor transfectants generated in a murine B cell lymphoma line migrated in chemotaxis assays in response to eotaxin, RANTES, and MCP-3, but not to any other chemokines tested. When expressed in several heterologous systems, the human receptor did not significantly bind to MIP-1 $\alpha$  under the conditions used. Moreover, chemotaxis and ligand binding assays using eosinophils and an eosinophilic-like cell line, indicate that RANTES and MCP-3 bind eosinophils through a receptor,

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which is distinct from C-C chemokine receptor 1, the MIP-1 $\alpha$ /RANTES receptor.

The role of MIP-1 $\alpha$  as an eosinophil chemoattractant has been controversial. Some investigators detect chemotactic responses (Rot, A., et al., *J. Exp. Med.*, 176: 1489-1495 (1995)), whereas others do not (Figure 4, Example 1; Ebisawa, M., et al., *J. Immunol.*, 153: 2153-2160 (1994); and Ponath, P.D., et al., *J. Clin. Invest.*, (1996) (in press)). Interestingly, MIP-1 $\alpha$  is an eosinophil chemoattractant in the mouse, and this appears to be mediated through the murine CKR-3 homologue, which also binds and signals with murine eotaxin (Post, T.W., et al., *J. Immunol.*, 155: 5299-5305 (1995); the teachings of which are incorporated herein by reference in their entirety).

Using the proteins and antibodies of the present invention, additional ligands, as well as additional cell types (e.g., leukocytes, such as basophils) which express CKR-3 receptor, can be identified. The ability of other chemokines to bind mammalian CKR-3 receptors can be assessed according to the present invention.

The cloning and characterization of clones encoding a novel receptor, and the isolation and characterization of the novel CKR-3 receptor which demonstrably binds and mediates chemotaxis in response to chemokines such as eotaxin, RANTES and MCP-3, suggests that this receptor is a member of a family of seven transmembrane spanning G protein-coupled receptors which are involved in selective leukocyte chemotaxis and activation in response to chemokines. The CKR-3 receptor and its mammalian homologs are distinct from the MIP-1 $\alpha$ /RANTES receptor and the MCP-1 receptor (i.e., are receptors other than C-C chemokine receptor 1 (CKR-1) and MCP-1 receptor (CKR-2) and their homologs).

Because of the role of chemokine receptors in the selective induction of leukocyte chemotaxis and leukocyte

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activation in response to chemoattractants, chemokine receptors play a fundamental role in leukocyte migration, and particularly in migration associated with inflammation. Chemokines, produced at sites of inflammation and  
5 infection, specifically recruit selected leukocyte subtypes from the circulation to the site of inflammation in the tissues. Subsequent to chemokine binding to a leukocyte chemokine receptor, integrin activation occurs, and leukocytes adhere firmly to the endothelial cell wall via  
10 leukocyte integrins and endothelial cell adhesion molecules. The leukocytes become flat in shape, and migrate through the endothelium towards sites of inflammation in the tissues. The specificity of a leukocyte for a tissue or inflammatory site is, in many  
15 cases, determined at the level of the chemokine-receptor interaction, rather than at the level of the adhesion interaction between integrin and cellular adhesion molecules.

RANTES and MCP-3 are among the most potent chemotactic  
20 cytokines for eosinophils and basophils. In addition, RANTES is reported to be a chemoattractant for memory T cells, a subpopulation of T lymphocytes. As shown herein, RANTES and MCP-3 can induce chemotaxis of eosinophils and eosinophil-like cells. CKR-3 receptor proteins described  
25 herein also bind RANTES and MCP-3 with high affinity.

As is further shown herein, CKR-3 binds eotaxin specifically and with high affinity (comparable to the binding affinity observed with eosinophils), and the CKR-3 receptor is highly restricted in its expression. Although  
30 a number of chemoattractants have been identified for eosinophils, such as RANTES and MCP-3 (Baggiolini, M. and Dahinden, C.A., *Immunol. Today*, 15: 127-33 (1994); Dahinden, C.A., et al., *J. Exp. Med.*, 179: 751-756 (1994); Kameyoshi, Y., et al., *J. Exp. Med.*, 176: 587-592 (1992);  
35 Rot, A., et al., *J. Exp. Med.*, 176: 1489-1495 (1995)), as

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well as PAF, C5a, and IL-16 (Wardlaw, A.J., et al., *J. Clin. Invest.*, 78: 1701-1706 (1986); Gerard, N.P., et al., *J. Biol. Chem.*, 264: 1760-1765 (1989); Rand, T.H., et al., *J. Exp. Med.*, 173: 1521-1528 (1991)), these chemoattractants  
5 also induce the migration of other leukocyte cell types. In contrast, the chemokine eotaxin, a potent eosinophil chemoattractant originally identified in guinea pigs and subsequently in mouse and human, is selectively chemotactic for eosinophils (Jose, P.J., et al., *Biochem. Biophys. Res.*  
10 *Commun.*, 205: 788-794 (1994); Jose, P.J., et al., *J. Exp. Med.*, 179: 881-887 (1994); Rothenburg, M.E. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 92: 8960-8964 (1995); Ponath, P.D., et al., *J. Clin. Invest.*, (1996)(in press)). In addition, eotaxin binds to and signals through CKR-3 with a  
15 high degree of fidelity, in contrast to chemokines such as MCP-3, which binds CKR-1 and CKR-2 (Ben-Baruch, A., et al., *J. Biol. Chem.*, 270: 22123-22128 (1995)) in addition to CKR-3, or MIP-1 $\alpha$ , which binds CKR-1 and CKR-4 (Neote, K., et al., *Cell*, 72: 415-425 (1993); Power, C.A., et al., *J.*  
20 *Biol. Chem.*, 270: 19495-19500 (1995)). The restricted expression of CKR-3 on eosinophils, and the fidelity of eotaxin binding to CKR-3, provides a potential mechanism for the selective recruitment and migration of eosinophils within tissues. In this regard, the production of eotaxin  
25 within a tissue can lead to selective eosinophil recruitment; eotaxin injection into the skin of rhesus monkeys leads to selective eosinophil migration. In addition, eotaxin was shown to recruit eosinophils *in vivo* at a 10-fold lower dose than RANTES, similar to the *in*  
30 *vitro* chemotaxis of CKR-3 transfectants (Ponath, P.D., et al., *J. Clin. Invest.*, (1996)(in press)).

Modulation of mammalian CKR-3 receptor function according to the present invention, through the inhibition or promotion of receptor function, such as binding,  
35 signalling or stimulation of a cellular response, provides

an effective and selective way of inhibiting or promoting leukocyte-mediated inflammatory action, particularly that of eosinophils and/or T cells. Ligands, inhibitors and promoters of CKR-3 receptor function, such as those  
5 identified as described herein, can be used to modulate leukocyte function for therapeutic purposes.

Eosinophils do not express the MIP-1 $\alpha$  receptor, and do not express significant amounts of MCP-1 receptor. In addition, as noted above, eotaxin and RANTES are some of  
10 the most potent chemoattractants for eosinophils, and eotaxin and RANTES bind specifically and with high affinity to the CKR-3 receptor. As a major eosinophil and lymphocyte chemokine receptor, the CKR-3 receptor is an important target for interfering with or promoting  
15 eosinophil and/or T lymphocyte function. Compounds which inhibit or promote CKR-3 receptor function, such as ligands, inhibitors and promoters identified according to the present method, are particularly useful for modulating eosinophil and/or T cell function for therapeutic purposes.

#### 20 Nucleic Acids, Constructs and Vectors

The present invention relates to isolated and/or recombinant (including, e.g., essentially pure) nucleic acids having sequences which encode a mammalian (e.g., human) receptor protein designated Eos L2 or C-C Chemokine  
25 Receptor 3 (CKR-3) or a portion of said receptor. In one embodiment, the nucleic acid or portion thereof encodes a protein or polypeptide having at least one function characteristic of a mammalian C-C chemokine receptor (e.g., a mammalian CKR-3 receptor), such as a binding activity  
30 (e.g., ligand, inhibitor and/or promoter binding), a signalling activity (e.g., activation of a mammalian G protein, induction of rapid and transient increase in the concentration of cytosolic free calcium [Ca<sup>2+</sup>]<sub>i</sub>), and/or

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stimulation of a cellular response (e.g., stimulation of chemotaxis, exocytosis or inflammatory mediator release by leukocytes, integrin activation). The present invention also relates more specifically to isolated and/or

5 recombinant nucleic acids or a portion thereof comprising sequences which encode a mammalian CKR-3 receptor or a portion thereof.

The invention further relates to isolated and/or recombinant nucleic acids that are characterized by (1)

10 their ability to hybridize to: (a) a nucleic acid having the sequence SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5, (b) a the complement of any one of SEQ ID NOS:1, 3 or 5, (c) a portion of the foregoing comprising the coding region (nucleotides 181-1245 of SEQ ID NO:1, nucleotides 92-1156

15 of SEQ ID NO:3, or nucleotides 15-1079 of SEQ ID NO:5), or the RNA counterpart of any one of the foregoing, wherein U is substituted for T; or (2) by their ability to encode a polypeptide having the amino acid sequence SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 or a functional equivalents thereof

20 (i.e., a polypeptide having ligand binding activity for one or more natural or physiological ligand(s) of the receptor and/or stimulatory function responsive to ligand binding, such that it can stimulate a cellular response (e.g., stimulation of chemotaxis, exocytosis or inflammatory

25 mediator release by leukocytes); or (3) by both characteristics.

In one embodiment, the percent amino acid sequence identity between SEQ ID NOS:2, 4 or 6 and functional equivalents thereof is at least about 70% ( $\geq 70\%$ ). In a

30 preferred embodiment, functional equivalents of SEQ ID NOS:2, 4 or 6 share at least about 80% sequence identity with SEQ ID NOS:2, 4 or 6, respectively. More preferably, the percent amino acid sequence identity between SEQ ID NOS:2, 4 or 6 and functional equivalents thereof is at

35 least about 90%, and still more preferably, at least about

95%. Isolated and/or recombinant nucleic acids meeting these criteria comprise nucleic acids having sequences identical to sequences of naturally occurring mammalian CKR-3 receptors and portions thereof, or variants of the naturally occurring sequences. Such variants include mutants differing by the addition, deletion or substitution of one or more residues, modified nucleic acids in which one or more residues is modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Such nucleic acids can be detected and isolated by hybridization under high stringency conditions or moderate stringency conditions, for example. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are explained on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. et al., eds., Vol. 1, Suppl. 26, 1991), the teachings of which are incorporated herein by reference (see also Example 2). Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Thus, high or moderate stringency conditions can be determined empirically, depending in part upon the characteristics of the known DNA to which other unknown nucleic acids are being compared for homology.

Isolated and/or recombinant nucleic acids that are characterized by their ability to hybridize to a nucleic acid having the sequence SEQ ID NOS: 1, 3 or 5 or the complements of any one of SEQ ID NOS: 1, 3 or 5 (e.g. under high or moderate stringency conditions) may further encode a protein or polypeptide having at least one function characteristic of a mammalian C-C chemokine receptor (e.g., a mammalian CKR-3 receptor), such as a binding activity (e.g., ligand, inhibitor and/or promoter binding), a

signalling activity (e.g., activation of a mammalian G protein, induction of rapid and transient increase in the concentration of cytosolic free calcium  $[Ca^{2+}]_i$ ), and/or stimulation of a cellular response (e.g., stimulation of chemotaxis, exocytosis or inflammatory mediator release by leukocytes, integrin activation).

The signalling function of a protein or polypeptide encoded by hybridizing nucleic acid can be detected by enzymatic assays for G protein activity responsive to receptor binding (e.g., exchange of GTP for GDP on the G protein  $\alpha$  subunit, using membrane fractions). G protein coupling can be further assessed, for example, using assays in which stimulation by G protein is blocked by treatment or pre-treatment of cells or a suitable cellular fraction (e.g., membranes) with specific inhibitors of G proteins, such as *Bordetella pertussis* toxin (Bischoff, S.C. et al., *Eur. J. Immunol.* 23: 761-767 (1993); Sozzani, S. et al., *J. Immunol.* 147: 2215-2221 (1991)).

The stimulatory function of a protein or polypeptide encoded by hybridizing nucleic acid can be detected by standard assays for chemotaxis or mediator release, using cells expressing the protein or polypeptide (e.g., assays which monitor chemotaxis, exocytosis (e.g., of enzymes such as eosinophil peroxidase,  $\beta$ -glucuronidase) or mediator release in response to a ligand (e.g., a chemokine such as eotaxin, RANTES or MCP-3) or a promoter.

The binding function of a protein or polypeptide encoded by hybridizing nucleic acid can be detected in binding or binding inhibition assays using membrane fractions containing receptor or cells expressing receptor, for instance (see e.g., Example 9; Van Riper et al., *J. Exp. Med.*, 177: 851-856 (1993); Sledziewski et al., U.S. Patent No. 5,284,746 (Feb. 8, 1994)). Thus, the ability of the encoded protein or polypeptide to bind a ligand, such



as eotaxin, RANTES or MCP-3, an inhibitor and/or promoter, can be assessed. Functions characteristic of a mammalian CKR-3 receptor may also be assessed by other suitable methods (see below).

5        These methods, alone or in combination with other suitable methods can also be used in procedures for the identification and/or isolation of nucleic acids which encode a polypeptide having the amino acid sequence SEQ ID NO: 2, 4, 6 or functional equivalents thereof, and having  
10       an activity detected by the assay. Portions of the isolated nucleic acids which encode polypeptide portions of SEQ ID NO: 2, 4 or 6 having a certain function can be also identified and isolated in this manner.

      Nucleic acids of the present invention can be used in  
15       the production of proteins or polypeptides. For example, a nucleic acid containing all or part of the coding sequence for a mammalian CKR-3 receptor, or DNA which hybridizes to the sequence SEQ ID NO: 1, 3 or 5, or the complement of any one of SEQ ID NO: 1, 3 or 5, can be incorporated into  
20       various constructs and vectors created for further manipulation of sequences or for production of the encoded polypeptide in suitable host cells.

      Nucleic acids referred to herein as "isolated" are nucleic acids separated away from the nucleic acids of the  
25       genomic DNA or cellular RNA of their source of origin (e.g., as it exists in cells or in a mixture of nucleic acids such as a library), and may have undergone further processing. "Isolated" nucleic acids include nucleic acids obtained by methods described herein, similar methods or  
30       other suitable methods, including essentially pure nucleic acids, nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods, and recombinant nucleic acids which are isolated. Nucleic acids referred to herein as "recombinant" are nucleic acids  
35       which have been produced by recombinant DNA methodology,

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including those nucleic acids that are generated by procedures which rely upon a method of artificial recombination, such as the polymerase chain reaction (PCR) and/or cloning into a vector using restriction enzymes.

- 5 "Recombinant" nucleic acids are also those that result from recombination events that occur through the natural mechanisms of cells, but are selected for after the introduction to the cells of nucleic acids designed to allow and make probable a desired recombination event.

10 Antisense Constructs

- In another embodiment, the nucleic acid is an antisense nucleic acid, which is complementary, in whole or in part, to a target molecule comprising a sense strand, and can hybridize with the target molecule. The target can  
15 be DNA, or its RNA counterpart (i.e., wherein T residues of the DNA are U residues in the RNA counterpart). When introduced into a cell using methods known in the art or other suitable methods, antisense nucleic acid can inhibit the expression of the gene encoded by the sense strand.  
20 Antisense nucleic acids can be produced by standard techniques.

- In one embodiment, the antisense nucleic acid is wholly or partially complementary to and can hybridize with a target nucleic acid, wherein the target nucleic acid can  
25 hybridize to a nucleic acid having the sequence of the complement of SEQ ID NO:1, 3 or 5. For example, antisense nucleic acid can be complementary to a target nucleic acid having the sequence of SEQ ID NO: 5 or a portion thereof sufficient to allow hybridization. In another embodiment,  
30 the antisense nucleic acid is wholly or partially complementary to and can hybridize with a target nucleic acid which encodes a mammalian CKR-3 receptor (e.g., human Eos L2 receptor).

Antisense nucleic acids are useful for a variety of purposes, including research and therapeutic applications. For example, a construct comprising an antisense nucleic acid can be introduced into a suitable cell to inhibit  
5 receptor expression. Such a cell provides a valuable control cell, for instance in assessing the specificity of receptor-ligand interaction with the parent cell or other related cell types. In another aspect, such a construct is introduced into some or all of the cells of a mammal. The  
10 antisense nucleic acid inhibits receptor expression, and inflammatory processes mediated by CKR-3 receptors in the cells containing the construct can be inhibited. Thus, an inflammatory disease or condition can be treated using an antisense nucleic acid of the present invention. Suitable  
15 laboratory animals comprising an antisense construct can also provide useful models for deficiencies of leukocyte function, and of eosinophil deficiency in particular, and provide further information regarding CKR-3 receptor function. Such animals can provide valuable models of  
20 infectious disease, useful for elucidating the role of leukocytes, such as eosinophils and/or T lymphocytes, in host defenses.

#### Mammalian Nucleic Acids

Because advances in the understanding and treatment of  
25 human inflammatory and autoimmune diseases and of parasitic infections would be of tremendous benefit, human CKR-3 was the species selected for most of the experimental work described herein. However, the approaches described to isolate and manipulate the genomic and cDNAs of human CKR-3  
30 (Eos L2), to construct vectors and host strains, and to produce and use the receptor or fragments thereof, can be applied to other mammalian species, including, but not limited to primate (e.g., a primate other than a human, such as a monkey (e.g., cynomolgus monkey)), bovine (e.g.,

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cows), ovine (e.g., sheep), equine (e.g., horses), canine (e.g., dog), feline (e.g., domestic cat) and rodent (e.g., guinea pig, murine species such as rat, mouse) species.

The human CKR-3 cDNA or genomic clones described here, or  
5 sufficient portions thereof, whether isolated and/or recombinant or synthetic, including fragments within the coding sequence produced by PCR, can be used as probes to detect and/or recover homologous CKR-3 genes (homologs) or other related receptor genes (e.g., novel C-C chemokine  
10 receptor genes) from other mammalian species (e.g., by hybridization, PCR or other suitable techniques). This can be achieved using the procedures described herein or other suitable methods.

#### Proteins and Peptides

15 The invention also relates to proteins or polypeptides encoded by nucleic acids of the present invention. The proteins and polypeptides of the present invention can be isolated and/or recombinant. Proteins or polypeptides referred to herein as "isolated" are proteins or  
20 polypeptides purified to a state beyond that in which they exist in mammalian cells. "Isolated" proteins or polypeptides include proteins or polypeptides obtained by methods described herein, similar methods or other suitable methods, including essentially pure proteins or  
25 polypeptides, proteins or polypeptides produced by chemical synthesis, or by combinations of biological and chemical methods, and recombinant proteins or polypeptides which are isolated. Proteins or polypeptides referred to herein as "recombinant" are proteins or polypeptides produced by the  
30 expression of recombinant nucleic acids.

In a preferred embodiment, the protein or polypeptide has at least one function characteristic of a mammalian CKR-3 receptor, such as a binding activity (e.g., ligand, inhibitor and/or promoter binding), a signalling activity

(e.g., activation of a mammalian G protein, induction of rapid and transient increase in the concentration of cytosolic free calcium  $[Ca^{2+}]_i$ ), and/or stimulation of a cellular response (e.g., stimulation of chemotaxis, exocytosis or inflammatory mediator release by leukocytes, integrin activation). As such, these proteins are referred to as CKR-3 proteins of mammalian origin or mammalian chemokine receptor 3 proteins, and include, for example, naturally occurring mammalian CKR-3 receptors, variants of those proteins and/or portions thereof. Such variants include polymorphic variants and natural or artificial mutants, differing by the addition, deletion or substitution of one or more amino acid residues, or modified polypeptides in which one or more residues is modified, and mutants comprising one or more modified residues. An example would be a mammalian CKR-3 receptor protein which binds eotaxin.

In a particularly preferred embodiment, like naturally occurring mammalian CKR-3 receptor proteins or polypeptides, the mammalian CKR-3 receptors of the present invention have ligand binding function for one or more natural or physiological ligand(s) and/or stimulatory function responsive to ligand binding, such that they can stimulate a cellular response (e.g., stimulation of chemotaxis, exocytosis or inflammatory mediator release by leukocytes). For example, in the case of a human chemokine receptor 3 protein, an isolated human CKR-3 protein will bind the one or more natural or physiological ligand(s). As shown herein, an isolated human CKR-3 protein binds eotaxin and RANTES specifically and with high affinity, and specifically binds MCP-3. In one embodiment, a human CKR-3 receptor protein or polypeptide also triggers chemotaxis, exocytosis or inflammatory mediator release by leukocytes in response to ligand binding.

The invention further relates to fusion proteins, comprising a mammalian CKR-3 receptor protein or polypeptide (as described above) as a first moiety, linked to a second moiety not occurring in the mammalian CKR-3 receptor as found in nature. Thus, the second moiety can be an amino acid or polypeptide. The first moiety can be in an N-terminal location, C-terminal location or internal to the fusion protein. In one embodiment, the fusion protein comprises a human CKR-3 receptor as the first moiety, and a second moiety comprising a linker sequence and affinity ligand (e.g., an enzyme, an antigen, epitope tag).

Fusion proteins can be produced by a variety of methods. For example, some embodiments can be produced by the insertion of a CKR-3 gene or portion thereof into a suitable expression vector, such as Bluescript®II SK +/- (Stratagene), pGEX-4T-2 (Pharmacia) and pET-15b (Novagen). The resulting construct is then introduced into a suitable host cell for expression. Upon expression, fusion protein can be isolated or purified from a cell lysate by means of a suitable affinity matrix (see e.g., *Current Protocols in Molecular Biology* (Ausubel, F.M. et al., eds., Vol. 2, Suppl. 26, pp. 16.4.1-16.7.8 (1991))). In addition, affinity labels provide a means of detecting CKR-3 receptor proteins or polypeptides present in a fusion protein. For example, the cell surface expression or presence in a particular cell fraction of a fusion protein comprising an antigen or epitope affinity label can be detected by means of an appropriate antibody (see, e.g., Example 3).

The invention also relates to isolated and/or recombinant portions of a CKR-3 receptor of mammalian origin, such as a fragment of a human CKR-3 receptor. As is described in more detail below, portions of a mammalian CKR-3 receptor can be produced (e.g., synthetic peptides) and used to produce antibodies. In one embodiment, an

isolated and/or recombinant portion (e.g., a peptide) of a selected mammalian CKR-3 receptor has at least one immunological property. As used herein, with reference to a portion of a receptor, an immunological property includes

5 immunoreactivity (bound by antibodies raised against a mammalian CKR-3 receptor protein of the present invention, including a portion thereof), immunogenicity (induces an antibody response against itself when used in a suitable immunization protocol), and/or cross-reactivity (induces

10 antibodies reactive with a selected mammalian receptor). Furthermore, portions of a CKR-3 receptor having at least one function characteristic of mammalian CKR-3 receptors, such as binding activity, signalling activity, or stimulatory function (stimulation of a cellular response),

15 can also be produced. Extensive studies on the structure and function of mammalian G protein-coupled receptors provide the basis for being able to divide mammalian CKR-3 receptors into functional domains (see e.g., Lefkowitz et al., *J. Biol. Chem.*, 263: 4993-4996 (1988); Panayotou and

20 Waterfield, *Curr. Opin. Cell Biol.*, 1: 167-176 (1989)). Furthermore, portions of the receptor can be produced which have full or partial function on their own, or which when joined with another portion of a second receptor (though fully, partially, or nonfunctional alone), constitute a

25 functional protein having at least one function characteristic of a mammalian CKR-3 receptor (e.g., ligand-, inhibitor- or promoter-binding function). (See, e.g., Sledziewski et al., U.S. Patent No. 5,284,746 regarding the construction and use of hybrid G

30 protein-coupled receptors useful in detecting the presence of ligand in a test sample).

Method of Producing Recombinant Mammalian CKR-3 Receptors

Another aspect of the invention relates to a method of producing a mammalian CKR-3 receptor or a portion thereof. Constructs suitable for the expression of a mammalian CKR-3 receptor or a portion thereof are also provided. The constructs can be introduced into a suitable host cell. Cells expressing a recombinant mammalian CKR-3 receptor or a portion thereof can be isolated and maintained in culture. Such cells are useful for a variety of purposes such as the production of protein for characterization, isolation and/or purification, and in binding assays for the detection of ligands, or inhibitors or promoters of ligand binding. Suitable host cells can be procaryotic, including bacterial cells such as *E. coli*, *B. subtilis* and or other suitable bacteria, or eucaryotic, such as fungal or yeast cells (e.g., *Pichia pastoris*, *Aspergillus species*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Neurospora crassa*), or other lower eucaryotic cells, and cells of higher eucaryotes such as those from insects (e.g., Sf9 insect cells) or mammals (e.g., 293 cells, Chinese hamster ovary cells (CHO)). (See, e.g., Ausubel, F.M. et al., eds. *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons Inc., (1993)).

Host cells which produce a recombinant mammalian CKR-3 receptor protein, portion thereof, or fusion protein can be produced as follows. A nucleic acid encoding all or part of the coding sequence for a mammalian CKR-3 receptor or fusion protein can be inserted into a nucleic acid vector, e.g., a DNA vector, such as a plasmid, virus or other suitable replicon for expression. A variety of vectors are available, including vectors which are maintained in single copy or multiple copy, or which become integrated into the host cell chromosome.



The transcriptional and/or translational signals of a selected CKR-3 receptor can be used to direct expression. Alternatively, suitable expression vectors are available. Suitable vectors for expression of a nucleic acid encoding  
5 all or part of the coding sequence for a mammalian CKR-3 receptor or fusion protein can contain a number of additional components, including, but not limited to one or more of the following: an origin of replication; a selectable marker gene; one or more expression control  
10 elements, such as a transcriptional control element (e.g., a promoter, an enhancer, terminator), and/or one or more translation signals; a signal sequence or leader sequence (for membrane targeting encoded by the vector or receptor).

A promoter is provided for expression in a suitable  
15 host cell. Promoters can be constitutive or inducible. In the vectors, the promoter is operably linked to a nucleic acid encoding the receptor protein, portion thereof or fusion protein, and is capable of directing expression of the encoded polypeptide. A variety of suitable promoters  
20 for procaryotic (e.g., lac, tac, T3, T7 promoters for *E. coli*) and eukaryotic (e.g., yeast alcohol dehydrogenase (ADH1), SV40, CMV) hosts are available.

In addition, the expression vectors typically comprise a selectable marker for selection of host cells carrying  
25 the vector and an origin of replication, in the case of replicable expression vector. Genes encoding products which confer antibiotic or drug resistance are common selectable markers and may be used in prokaryotic (e.g.,  $\beta$ -lactamase gene (ampicillin resistance), Tet gene for  
30 tetracycline resistance) and eukaryotic cells (e.g., neomycin (G418 or geneticin), gpt (mycophenolic acid), ampicillin, or hygromycin resistance genes). Dihydrofolate reductase marker genes permit selection with methotrexate in a variety of hosts. Genes encoding the gene product of  
35 auxotrophic markers of the host (e.g., *LEU2*, *URA3*, *HIS3*)

are often used as selectable markers in yeast. Use of viral (e.g., baculovirus) or phage vectors, and vectors which are capable of integrating into the genome of the host cell, such as retroviral vectors, are also contemplated. The present invention also relates to cells carrying these expression vectors.

When the nucleic acid encoding the receptor protein or polypeptide is inserted into the vector, operably linked to one or more of these components, and the resulting construct is introduced into host cells maintained under conditions suitable for expression, the receptor protein or polypeptide is produced. The construct can be introduced into cells by a method appropriate to the host cell selected (e.g., transformation, transfection, electroporation, infection). For production of receptor, host cells comprising the construct are maintained under conditions appropriate for expression, e.g., in the presence of inducer (e.g., n-butyrate), suitable media supplemented with appropriate salts, growth factors, antibiotic, nutritional supplements, etc.

#### Antibodies

The invention further relates to antibodies reactive with a CKR-3 receptor or portion thereof. In one embodiment, antibodies are raised against an isolated and/or recombinant mammalian CKR-3 protein including portions thereof (e.g., a peptide). In a preferred embodiment, the antibodies specifically bind CKR-3 receptor(s) or a portion thereof.

The antibodies of the present invention can be polyclonal or monoclonal (see e.g., Example 5), and the term antibody is intended to encompass both polyclonal and monoclonal antibodies. Antibodies of the present invention can be raised against an appropriate immunogen, including proteins or polypeptides of the present invention, such as

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isolated and/or recombinant mammalian CKR-3 receptor protein or portion thereof, or synthetic molecules, such as synthetic peptides. In addition, cells which express receptor, such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor. See for example, Chuntharapai et al., *J. Immunol.* 152: 1783-1789 (1994)).

Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler et al., *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein et al., *Nature* 266: 550-552 (1977); Koprowski et al., U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. et al., Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0) with antibody producing cells. The antibody producing cell, preferably those of the spleen or lymph nodes, are obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) are isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity are selected by a suitable assay (e.g., ELISA).

Single chain antibodies, and chimeric, humanized or primatized (CDR-grafted) antibodies, as well as chimeric or CDR-grafted single chain antibodies, comprising portions derived from different species, are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared

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as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly et al., U.S. Patent No.

- 5 4,816,567; Cabilly et al., European Patent No. 0,125,023 B1; Boss et al., U.S. Patent No. 4,816,397; Boss et al., European Patent No. 0,120,694 B1; Neuberger, M.S. et al., WO 86/01533; Neuberger, M.S. et al., European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; and
- 10 Winter, European Patent No. 0,239,400 B1. See also, Newman, R. et al., *BioTechnology*, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner et al., U.S. Patent No. 4,946,778 and Bird, R.E. et al., *Science*, 242: 423-426 (1988)) regarding single chain antibodies.
- 15 In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced. Functional fragments of foregoing antibodies retain at least one binding function and/or modulation function of the full-
- 20 length antibody from which they are derived. For example, antibody fragments capable of binding to a mammalian CKR-3 receptor or portion thereof, including, but not limited to, Fv, Fab, Fab' and F(ab')<sub>2</sub> fragments are encompassed by the invention. Such fragments can be produced by enzymatic
- 25 cleavage or by recombinant techniques. For instance, papain or pepsin cleavage can generate Fab or F(ab')<sub>2</sub> fragments, respectively. Alternatively, antibodies can be produced in a variety of truncated forms using antibody genes in which one or more stop codons has been introduced
- 30 upstream of the natural stop site. For example, a chimeric gene encoding a F(ab')<sub>2</sub> heavy chain portion can be designed to include DNA sequences encoding the CH<sub>1</sub> domain and hinge region of the heavy chain.

The antibodies of the present invention are useful in a variety of applications, including research, diagnostic and therapeutic applications. For instance, they can be used to isolate and/or purify receptor or portions thereof, and to study receptor structure (e.g., conformation) and function.

The antibodies of the present invention can also be used to modulate receptor function in research and therapeutic applications. For instance, antibodies can act as inhibitors to inhibit (reduce or prevent) (a) binding (e.g., of a ligand, a second inhibitor or a promoter) to the receptor, (b) a receptor signalling, (c) and/or a stimulatory function. Antibodies which act as inhibitors of receptor function can block ligand or promoter binding directly or indirectly (e.g., by causing a conformational change). For example, antibodies can inhibit receptor function by inhibiting binding of a ligand, or by desensitization (with or without inhibition of binding of a ligand).

Antibodies which bind receptor can also act as agonists of receptor function, triggering or stimulating a receptor function, such as a signalling and/or a stimulatory function of a receptor (e.g., chemotaxis, exocytosis or pro-inflammatory mediator release) upon binding to receptor.

In addition, the various antibodies of the present invention can be used to detect or measure the expression of receptor, for example, on leukocytes such as eosinophils, basophils, and lymphocytes, or on cells transfected with a receptor gene. Thus, they also have utility in applications such as cell sorting (e.g., flow cytometry, fluorescence activated cell sorting), for diagnostic or research purposes.

Anti-idiotypic antibodies are also provided. Anti-idiotypic antibodies recognize antigenic determinants

associated with the antigen-binding site of another antibody. Anti-idiotypic antibodies can be prepared against second antibody by immunizing an animal of the same species, and preferably of the same strain, as the animal  
5 used to produce the second antibody. See e.g., U.S. Patent No. 4,699,880.

In one embodiment, antibodies are raised against receptor or a portion thereof, and these antibodies are used in turn to produce an anti-idiotypic antibody. The  
10 anti-Id produced thereby can bind compounds which bind receptor, such as ligands, inhibitors or promoters of receptor function, and can be used in an immunoassay to detect or identify or quantitate such compounds. Such an anti-idiotypic antibody can also be an inhibitor of  
15 receptor function, although it does not bind receptor itself.

Anti-idiotypic (i.e., Anti-Id) antibody can itself be used to raise an anti-idiotypic antibody (i.e., Anti-anti-Id). Such an antibody can be similar or  
20 identical in specificity to the original immunizing antibody. In one embodiment, antibody antagonists which block binding to receptor can be used to raise Anti-Id, and the Anti-Id can be used to raise Anti-anti-Id, which can have a specificity which is similar or identical to that of  
25 the antibody antagonist. These anti-anti-Id antibodies can be assessed for inhibitory effect on receptor function to determine if they are antagonists.

Single chain, and chimeric, humanized or primatized (CDR-grafted), as well as chimeric or CDR-grafted single  
30 chain anti-idiotypic antibodies can be prepared, and are encompassed by the term anti-idiotypic antibody. Antibody fragments of such antibodies can also be prepared.

Identification of Ligands, Inhibitors or Promoters of  
Receptor Function

As used herein, a ligand is a substance which binds to a receptor protein. A ligand of a selected mammalian CKR-3  
5 receptor is a substance which binds to the selected mammalian receptor. In one embodiment, a ligand can bind selectively to two or more mammalian chemokine receptors, including CKR-3. In a preferred embodiment, ligand binding of a mammalian CKR-3 receptor occurs with high affinity.  
10 The term ligand refers to substances including, but not limited to, a natural ligand, whether isolated and/or purified, synthetic, and/or recombinant, a homolog of a natural ligand (e.g., from another mammal), antibodies, portions of such molecules, and other substances which bind  
15 receptor. A natural ligand of a selected mammalian receptor can bind to the receptor under physiological conditions, and is of a mammalian origin which is the same as that of the mammalian CKR-3 receptor. The term ligand encompasses substances which are inhibitors or promoters of  
20 receptor activity, as well as substances which bind but lack inhibitor or promoter activity.

As used herein, an inhibitor is a substance which inhibits at least one function characteristic of a mammalian C-C chemokine receptor (e.g., a mammalian CKR-3  
25 receptor), such as a binding activity (e.g., ligand, inhibitor and/or promoter binding), a signalling activity (e.g., activation of a mammalian G protein, induction of rapid and transient increase in the concentration of cytosolic free calcium [ $\text{Ca}^{2+}$ ]<sub>i</sub>), and/or stimulation of a  
30 cellular response. The term inhibitor refers to substances including antagonists which bind receptor (e.g., an antibody, a mutant of a natural ligand, other competitive inhibitors of ligand binding), and substances which inhibit

receptor function without binding thereto (e.g., an anti-idiotypic antibody).

As used herein, a promoter is a substance which promotes (induces or enhances) at least one function characteristic of a mammalian C-C chemokine receptor (e.g., a mammalian CKR-3 receptor), such as a binding activity (e.g., ligand, inhibitor and/or promoter binding), a signalling activity (e.g., activation of a mammalian G protein, induction of rapid and transient increase in the concentration of cytosolic free calcium [ $\text{Ca}^{2+}$ ]<sub>i</sub>), and/or stimulation of a cellular response. The term promoter refers to substances including agonists which bind receptor (e.g., an antibody, a homolog of a natural ligand from another species), and substances which promote receptor function without binding thereto (e.g., by activating an associated protein).

The assays described below, which rely upon the nucleic acids and proteins of the present invention, can be used, alone or in combination with each other or other suitable methods, to identify ligands, inhibitors or promoters of a mammalian CKR-3 receptor protein or polypeptide. Human CKR-3 does not usually exist in cells at levels suitable for high-throughput screening; thus, cells which contain and express a nucleic acid of the present invention are particularly valuable in identifying ligands, inhibitors and promoters of CKR-3 receptor proteins.

Upon isolation of a CKR-3 receptor gene from a mammal, the gene can be incorporated into an expression system to produce a receptor protein or polypeptide as described above. An isolated and/or recombinant receptor protein or polypeptide, such as a receptor expressed in cells stably or transiently transfected with a construct comprising a nucleic acid of the present invention, or in a cell



fraction (e.g., membrane fraction from transfected cells) containing receptor, can be used in tests for receptor function. The receptor can be further purified if desired. Testing of receptor function can be carried out *in vitro* or  
5 *in vivo*.

An isolated, recombinant mammalian CKR-3 receptor protein, such as a human CKR-3 receptor as that shown in Figure 1A-1C (see also, SEQ ID NO:2), Figure 2A-2B (see also, SEQ ID NO:4) or SEQ ID NO:6, can be used in the  
10 present method, in which the effect of a compound is assessed by monitoring receptor function as described herein or using other suitable techniques. For example, stable or transient transfectants, such as A31/293/#20 stable transfectants (see e.g., Example 9), stable  
15 transfectants of mouse L1-2 pre-B cells (see e.g., Example 3), baculovirus infected Sf9 cells (see e.g., Example 4), can be used in binding assays. Stable transfectants of mouse L1-2 pre-B cells or of other suitable cells capable of chemotaxis can be used (see e.g., Example 3) in  
20 chemotaxis assays, for example.

According to the method of the present invention, compounds can be individually screened or one or more compounds can be tested simultaneously according to the methods herein. Where a mixture of compounds is tested,  
25 the compounds selected by the processes described can be separated (as appropriate) and identified by suitable methods (e.g., PCR, sequencing, chromatography). The presence of one or more compounds (e.g., a ligand, inhibitor, promoter) in a test sample can also be  
30 determined according to these methods.

Large combinatorial libraries of compounds (e.g., organic compounds, recombinant or synthetic peptides, "peptoids", nucleic acids) produced by combinatorial chemical synthesis or other methods can be tested (see  
35 e.g., Zuckerman, R.N. et al., *J. Med. Chem.*, 37: 2678-2685

(1994) and references cited therein; see also, Ohlmeyer, M.H.J. et al., *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993) and DeWitt, S.H. et al., *Proc. Natl. Acad. Sci. USA* 90:6909-6913 (1993), relating to tagged compounds; Rutter, W.J. et al. U.S. Patent No. 5,010,175; Huebner, V.D. et al., U.S. Patent No. 5,182,366; and Geysen, H.M., U.S. Patent No. 4,833,092). Where compounds selected from a combinatorial library by the present method carry unique tags, identification of individual compounds by chromatographic methods is possible.

In one embodiment, phage display methodology is used. For example, receptor is contacted with a phage (e.g., a phage or collection of phage such as a library) displaying a polypeptide under conditions appropriate for receptor binding (e.g., in a suitable binding buffer). Phage bound to receptor is selected using standard techniques or other suitable methods. Phage can be separated from receptor using a suitable elution buffer. For example, a change in the ionic strength or pH can lead to a release of phage. Alternatively, the elution buffer can comprise a release component or components designed to disrupt binding of compounds (e.g., one or more compounds which can disrupt binding of the displayed peptide to the receptor, such as a ligand, inhibitor, and/or promoter which competitively inhibits binding). Optionally, the selection process can be repeated or another selection step can be used to further enrich for phage which bind receptor. The displayed polypeptide is characterized (e.g., by sequencing phage DNA). The polypeptides identified can be produced and further tested for ligand binding, inhibitor and/or promoter function. Analogs of such peptides can be produced which will have increased stability or other desirable properties.

In one embodiment, phage expressing and displaying a fusion proteins comprising a coat protein with an N-

terminal peptide encoded by random sequence nucleic acids can be produced. Suitable host cells expressing a receptor protein or polypeptide of the present invention are contacted with the phage, bound phage are selected, recovered and characterized. (See e.g., Doorbar, J. and G. Winter, *J. Mol. Biol.*, 244: 361 (1994) discussing a phage display procedure used with a G protein-coupled receptor).

Other sources of potential ligands, inhibitors and/or promoters of a mammalian CKR-3 receptor include, but are not limited to, substances such as other chemoattractants (e.g., anaphylatoxin C5a and bacterial formylated tripeptide (fMLP)); other chemokines (e.g., eotaxin), such as a mammalian chemokine from the same mammal as the receptor, from another mammal (e.g., for a human receptor, a homolog of a human chemokine obtained from a non-human source); variants of other chemoattractants or chemokines, such as naturally occurring, synthetic or recombinant variants; other mammalian CKR-3 receptor ligands, inhibitors and/or promoters (e.g., antibodies, antagonists, agonists), and variants thereof; other G-protein coupled receptor ligands, inhibitors and/or promoters (e.g., antagonists or agonists); and soluble portions of a mammalian CKR-3 receptor, such as a suitable receptor peptide or analog which can inhibit receptor function (see e.g., Murphy, R.B., WO 94/05695).

The in vitro method of the present invention can be used in high-throughput screening. These assays can be adapted for processing large numbers of samples (e.g., a 96 well format). For such screening, use of a host cell expressing receptor, instead of isolated eosinophils, is preferred because of the difficulty in isolating eosinophils.

For binding assays, high level expression of receptor in a suitable host cell is preferred. Expression of receptor can be monitored in a variety of ways. For

instance, expression can be monitored using antibodies of the present invention which bind receptor or a portion thereof. Also, commercially available antibodies can be used to detect expression of an antigen- or epitope-tagged fusion protein comprising a receptor protein or polypeptide (e.g., FLAG tagged receptors; see Example 3).

#### Binding Assays

The isolated and/or recombinant receptor proteins, portions thereof, or suitable fusion proteins of the present invention, can be used in a method to select and identify compounds which bind to a (one or more) mammalian CKR-3 receptor protein, such as human CKR-3 receptor, and which are ligands, or potential inhibitors or promoters of receptor activity. Compounds selected by the method, including ligands, inhibitors or promoters, can be further assessed for an inhibitory or stimulatory effect on receptor function and/or for therapeutic utility.

In one embodiment, compounds which bind to an active, isolated and/or recombinant mammalian CKR-3 receptor protein or polypeptide are identified by the method. In this embodiment, the receptor protein or polypeptide used has at least one function characteristic of a CKR-3 receptor, such as a signalling activity (e.g., activation of a mammalian G protein), stimulatory function (e.g., stimulation of chemotaxis or inflammatory mediator release), and/or binding function (e.g., ligand, inhibitor and/or promoter binding). In a particularly preferred embodiment, the isolated and/or recombinant mammalian CKR-3 receptor protein or polypeptide has ligand binding function, such that it binds a natural ligand of the receptor.

For example, an isolated and/or recombinant mammalian CKR-3 receptor protein or polypeptide can be maintained under conditions suitable for binding, the receptor is

contacted with a compound to be tested, and binding is detected or measured. In one embodiment, a receptor protein can be expressed in cells stably or transiently transfected with a construct comprising a nucleic acid sequence which encodes a receptor of the present invention. The cells are maintained under conditions appropriate for expression of receptor. The cells are contacted with a compound under conditions suitable for binding (e.g., in a suitable binding buffer), and binding is detected by standard techniques. To measure binding, the extent of binding can be determined relative to a suitable control (e.g., compared with background determined in the absence of compound, compared with binding of a second compound (i.e., a standard), compared with binding of compound to untransfected cells). Optionally, a cellular fraction, such as a membrane fraction, containing receptor can be used in lieu of whole cells (see e.g., Example 9).

In one embodiment, the compound is labeled with a suitable label (e.g., fluorescent label, isotope label), and binding is determined by detection of the label. Specificity of binding can be assessed by competition or displacement, for example, using unlabeled compound or a second ligand as competitor.

Ligands of the mammalian receptor, including natural ligands from the same mammalian species or from another species, can be identified in this manner. The binding activity of a promoter or inhibitor which binds receptor can also be assessed using such a ligand binding assay.

Binding inhibition assays can also be used to identify ligands, and inhibitors and promoters which bind receptor and inhibit binding of another compound such as a ligand. For example, a binding assay can be conducted in which a reduction in the binding of a first compound (in the absence of a second compound), as compared binding of the first compound in the presence of the second compound, is

detected or measured. The receptor can be contacted with the first and second compounds simultaneously, or one after the other, in either order. A reduction in the extent of binding of the first compound in the presence of the second compound, is indicative of inhibition of binding by the second compound. For example, binding of the first compound could be decreased or abolished.

In one embodiment, direct inhibition of the binding of a first compound (e.g., a chemokine such as RANTES) to a human CKR-3 receptor by a second test compound is monitored. For example, the ability of a compound to inhibit the binding of  $^{125}\text{I}$ -labeled RANTES or  $^{125}\text{I}$ -labeled MCP-3 to human CKR-3 can be monitored. Such an assay can be conducted using either whole cells (e.g., butyric acid-differentiated HL-60 cells, or a suitable cell line containing nucleic acid encoding a human CKR-3 receptor) or a membrane fraction from said cells, for instance.

Other methods of identifying the presence of a compound(s) which bind a receptor are available, such as methods which monitor events which are triggered by receptor binding, including signalling function and/or stimulation of a cellular response (See below).

It will be understood that the inhibitory effect of antibodies of the present invention can be assessed in a binding inhibition assay. Competition between antibodies for receptor binding can also be assessed in the method in which the first compound in the assay is another antibody, under conditions suitable for antibody binding.

Ligands, as well as receptor-binding inhibitors (e.g., antagonists) and promoters (e.g., agonists), which are identified in this manner, can be further assessed to determine whether, subsequent to binding, they act to inhibit or activate other functions of CKR-3 receptors and/or to assess their therapeutic utility.

Signalling Assays

The binding of a ligand or promoter, such as an agonist, can result in signalling by a G protein-coupled receptor, and the activity of G proteins is stimulated.

- 5 The induction of induce signalling function by a compound can be monitored using any suitable method. For example, G protein activity, such as hydrolysis of GTP to GDP, or later signalling events triggered by receptor binding, such as induction of rapid and transient increase in the
- 10 concentration of intracellular (cytosolic) free calcium  $[Ca^{2+}]_i$ , can be assayed by methods known in the art or other suitable methods (see e.g., Neote, K. et al., *Cell*, 72: 415-425 1993); Van Riper et al., *J. Exp. Med.*, 177: 851-856 (1993); Dahinden, C.A. et al., *J. Exp. Med.*, 179: 751-756 (1994).

- 15 The functional assay of Sledziewski et al. using hybrid G protein coupled receptors can also be used to monitor the ability a ligand or promoter to bind receptor and activate a G protein (Sledziewski et al., U.S. Patent
- 20 No. 5,284,746, the teachings of which are incorporated herein by reference).

- A biological response of the host cell (triggered by binding to hybrid receptor) is monitored, detection of the response being indicative of the presence of ligand in the
- 25 test sample. Sledziewski et al. describes a method of detecting the presence of a ligand in a test sample, wherein the ligand is a compound which is capable of being bound by the ligand-binding domain of a receptor. In one embodiment of the method, yeast host cells are transformed
- 30 with a DNA construct capable of directing the expression of a biologically active hybrid G protein-coupled receptor (i.e., a fusion protein). The hybrid receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a

corresponding domain of a yeast G protein-coupled receptor, such as a STE2 gene product. The yeast host cells containing the construct are maintained under conditions in which the hybrid receptor is expressed, and the cells are  
5 contacted with a test sample under conditions suitable to permit binding of ligand to the hybrid receptor. The assay is conducted as described and the biological response of the host cell (triggered by binding to hybrid receptor) is monitored, detection of the response being indicative of a  
10 signalling function.

For instance, an assay is provided in which binding to a hybrid receptor derived from STE2 gene product leads to induction of the BAR1 promoter. Induction of the promoter is measured by means of a reporter gene ( $\beta$ -gal), which is  
15 linked to the BAR1 promoter and introduced into host cells on a second construct. Expression of the reporter gene can be detected by an in vitro enzyme assay on cell lysates or by the presence of blue colonies on plates containing an indicator (X-gal) in the medium, for example.

20 In another embodiment, the assay is used to identify potential inhibitors of receptor function. The inhibitory activity of a compound can be determined using a ligand or promoter in the assay, and assessing the ability of the compound to inhibit the activity induced by ligand or  
25 promoter.

Variants of known ligands can also be screened for reduced ability (decreased ability or no ability) to stimulate activity of a coupled G protein. In this  
30 embodiment, although the compound has ligand binding activity (as determined by another method in advance or later), engagement of the receptor does not trigger or only weakly triggers activity of a coupled G protein. Such compounds are potential antagonists, and can be further assessed using a suitable assay. For instance, the same  
35 assay can be conducted in the presence of a ligand or



promoter, and the ability of the compound to inhibit the activity of a ligand or promoter is assessed.

#### Chemotaxis and Assays of Cellular Stimulation

Chemotaxis assays can also be used to assess receptor  
5 function. These assays are based on the functional  
migration of cells *in vitro* or *in vivo* induced by a  
compound, and can be used to assess the binding and/or  
chemoattractant effect of ligands, inhibitors, or  
promoters. The use of an *in vitro* transendothelial  
10 chemotaxis assay is described in Example 1. Springer et  
*al.* describe a transendothelial lymphocyte chemotaxis assay  
(Springer et *al.*, WO 94/20142, published September 15,  
1994, the teachings of which are incorporated herein by  
reference; see also Berman et *al.*, *Immunol Invest.* 17: 625-  
15 677 (1988)). Migration across endothelium into collagen  
gels has also been described (Kavanaugh et *al.*, *J. Immunol.*,  
146: 4149-4156 (1991)).

Stable transfectants of mouse L1-2 pre-B cells or of  
other suitable host cells capable of chemotaxis can be used  
20 (see e.g., Example 3) in chemotaxis assays, for example.  
As is further described herein eosinophilic-like cell  
lines, such as the butyrate differentiated HL60 line which  
elaborates a CKR-3 receptor, can also be incorporated into  
chemotaxis assays.

25 Generally, chemotaxis assays monitor the directional  
movement or migration of a suitable cell (such as a  
leukocyte (e.g., lymphocyte, eosinophil, basophil)) into or  
through a barrier (e.g., endothelium, a filter), toward  
increased levels of a compound, from a first surface of the  
30 barrier toward an opposite second surface. Membranes or  
filters provide convenient barriers, such that the  
directional movement or migration of a suitable cell into  
or through a filter, toward increased levels of a compound,  
from a first surface of the filter toward an opposite

second surface of the filter, is monitored. In some assays, the membrane is coated with a substance to facilitate adhesion, such as ICAM-1, fibronectin or collagen.

5       For example, one can detect or measure the migration of cells in a suitable container (a containing means), from a first chamber into or through a microporous membrane into a second chamber which contains a compound to be tested, and which is divided from the first chamber by the  
10 membrane. A suitable membrane, having a suitable pore size for monitoring specific migration in response to compound, including, for example, nitrocellulose, polycarbonate, is selected. For example, pore sizes of about 3-8 microns, and preferably about 5-8 microns can be used. Pore size  
15 can be uniform on a filter or within a range of suitable pore sizes.

To assess migration, the distance of migration into the filter, the number of cells crossing the filter that remain adherent to the second surface of the filter, and/or  
20 the number of cells that accumulate in the second chamber can be determined using standard techniques (e.g., microscopy). In one embodiment, the cells are labeled with a detectable label (e.g., radioisotope, fluorescent label, antigen or epitope label), and migration can be assessed by  
25 determining the presence of the label adherent to the membrane and/or present in the second chamber using an appropriate method (e.g., by detecting radioactivity, fluorescence, immunoassay). The extent of migration induced by a compound can be determined relative to a  
30 suitable control (e.g., compared to background migration determined in the absence of the compound, to the extent of migration induced by a second compound (i.e., a standard), compared with migration of untransfected cells induced by the compound).

Chambers can be formed from various solids, such as plastic, glass, polypropylene, polystyrene, etc. Membranes which are detachable from the chambers, such as a Biocoat (Collaborative Biomedical Products) or Transwell (Costar, Cambridge, MA) culture insert, facilitate counting adherent cells.

In the container, the filter is situated so as to be in contact with fluid containing cells in the first chamber, and the fluid in the second chamber. Other than the test compound or additional ligand, inhibitor, or promoter present for the purpose of the assay, the fluid on either side of the membrane is preferably the same or substantially similar. The fluid in the chambers can comprise protein solutions (e.g., bovine serum albumin, fetal calf serum, human serum albumin) which may act to increase stability and inhibit nonspecific binding of cells, and/or culture media.

In a preferred embodiment, particularly for eosinophils, eosinophil-like cells, lymphocytes, or cells expressing a CKR-3 receptor, transendothelial migration is monitored. A transendothelial migration assay is preferred. Such assays are better physiological models, because they more accurately recapitulate in vivo conditions in which leukocytes emigrate from blood vessels toward chemoattractants present in the tissues at sites of inflammation by crossing the endothelial cell layer lining the vessel wall. In addition, transendothelial assays have lower background (signal to noise ratio).

In this embodiment, transmigration through an endothelial cell layer assessed. To prepare the cell layer, endothelial cells can be cultured on a microporous filter or membrane, optionally coated with a substance such as collagen, fibronectin, or other extracellular matrix proteins, to facilitate the attachment of endothelial cells. Preferably, endothelial cells are cultured until a

confluent monolayer is formed. A variety of mammalian endothelial cells can be available for monolayer formation, including for example, vein, artery or microvascular endothelium, such as human umbilical vein endothelial cells (Clonetics Corp, San Diego, CA) or a suitable cell line, such as the ECV 304 cell line used in Example 1. To assay chemotaxis in response to a particular mammalian receptor, endothelial cells of the same mammal are preferred; however endothelial cells from a heterologous mammalian species or genus can also be used.

Generally, the assay is performed by detecting the directional migration of cells into or through a membrane or filter, in a direction toward increased levels of a compound, from a first surface of the filter toward an opposite second surface of the filter, wherein the filter contains an endothelial cell layer on a first surface. Directional migration occurs from the area adjacent to the first surface, into or through the membrane, towards a compound situated on the opposite side of the filter. The concentration of compound present in the area adjacent to the second surface, is greater than that in the area adjacent to the first surface.

In one embodiment, a chemotaxis is used to test for ligand or promoter activity of a compound, a composition comprising cells capable of migration and expressing a mammalian CKR-3 receptor are placed in the first chamber, and a composition comprising the compound to be tested is placed in the second chamber, preferably in the absence of other ligands or promoters capable of inducing chemotaxis of the cells in the first chamber (having chemoattractant function). However, one or more ligands or promoters having chemoattractant function may be present. Compounds which can bind receptor and induce chemotaxis of the cells expressing a mammalian CKR-3 receptor in this assay are ligands or promoters of receptor function.

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In one embodiment used to test for an inhibitor, a composition comprising cells capable of migration and expressing a mammalian CKR-3 receptor are placed in the first chamber. A composition comprising one or more  
5 ligands or promoters capable of inducing chemotaxis of the cells in the first chamber (having chemoattractant function) is placed in the second chamber. Either shortly before the cells are placed in the first chamber, or simultaneously with the cells, a composition comprising the  
10 compound to be tested is placed, preferably, in the first chamber. Compounds which can bind receptor and inhibit the induction of chemotaxis, by a ligand or promoter, of the cells expressing a mammalian CKR-3 receptor in this assay are inhibitors of receptor function (i.e., inhibitors of  
15 stimulatory function). A reduction in the extent of migration induced by the ligand or promoter in the presence of the test compound, is indicative of inhibitory activity. (see e.g., Example 5). Separate binding studies (see  
above) could be performed to determine whether inhibition  
20 is a result of binding of the test compound to receptor or occurs via a different mechanism.

In vivo assays which monitor leukocyte infiltration of a tissue, in response to injection of a compound in the tissue, are described below (see Models of Inflammation).  
25 These models measure the ability of cells to respond to a ligand or promoter by emigration and chemotaxis to a site of inflammation.

In addition to the methods described, the effects of a ligand, inhibitor or promoter on the stimulatory function  
30 of the receptor can be assessed by monitoring cellular responses induced by active receptor, using suitable host cells containing receptor. Similarly, these assays can be used to determine the function of a receptor. For instance, exocytosis (e.g., degranulation of eosinophils  
35 leading to release of eosinophil cationic protein and/or

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one or more enzymes, or other granule components; release of histamine from basophils), inflammatory mediator release (such as release of bioactive lipids such as leukotrienes (e.g., leukotriene C<sub>4</sub>)), and respiratory burst (Rot, A. et al., *J. Exp. Med.*, 176: 1489-1495 (1992)), can be monitored by methods known in the art or other suitable methods. See e.g., Bischoff, S.C. et al., *Eur. J. Immunol.*, 23: 761-767 (1993) and Baggiolini, M. and C.A. Dahinden, *Immunology Today*, 15: 127-133 (1994) and references cited therein).

10 In one embodiment, a ligand, inhibitor and/or promoter is identified by monitoring the release of an enzyme upon degranulation or exocytosis by a cell capable of this function. Cells containing a nucleic acid of the present invention, which encodes an active receptor protein capable of stimulating exocytosis or degranulation are maintained in a suitable medium under suitable conditions, whereby receptor is expressed and degranulation can be induced. 15 The receptor is contacted with a compound to be tested, and enzyme release is assessed. The release of an enzyme into the medium can be detected or measured using a suitable assay, such as in an immunological assay, or biochemical assay for enzyme activity. 20

The medium can be assayed directly, by introducing components of the assay (e.g., substrate, co-factors, 25 antibody) into the medium (e.g., before, simultaneous with or after the cells and compound are combined). Alternatively, the assay can be performed on medium which has been separated from the cells or further fractionated prior to assay.

30 For example, convenient assays for are available for enzymes such as  $\beta$ -glucuronidase and eosinophil peroxidase (White, S.R. et al., A kinetic assay for eosinophil peroxidase activity in eosinophils and eosinophil

conditioned media, *J. Immunol. Methods*, 144(2): 257-63 (1991)).

Stimulation of degranulation by a compound can be indicative that the compound is a ligand or promoter of a mammalian CKR-3 receptor. In another embodiment, inhibition of degranulation is indicative of an inhibitor. In this embodiment, the cells expressing receptor are combined with a ligand or promoter, and a compound to be tested is added before, after or simultaneous therewith.

#### 10 Models of Inflammation

A variety of in vivo models of inflammation are available, which can be used to assess the effects of ligands, inhibitors, or promoters in vivo as therapeutic agents.

For example, primate models with eosinophilic infiltration to the lung, are available for in vivo testing (see e.g., Wegner, C.D. et al., *Science*, 247: 456 (1990)). In one embodiment, an antibody (e.g., a monoclonal antibody) which reacts with human CKR-3, and which cross-reacts with primate CKR-3, is administered to the animal. A number of parameters can be measured to assess in vivo efficacy including, but not limited to, the number of eosinophils in broncoalveolar lavage fluid, respiratory compliance, and respiratory rate. A decrease in symptoms of airway hypersensitivity is indicative of therapeutic benefit.

In addition, a sheep model for asthma, a guinea pig model for passive cutaneous anaphylaxis, or other suitable model can be used to assess compounds in vivo (see e.g., Weg, V.B. et al., *J. Exp. Med.*, 177: 561 (1993); Abraham, W.M. et al., *J. Clin. Invest.*, 93: 776 (1994)).

In addition, leukocyte infiltration upon intradermal injection of a compound into a suitable animal, such as rabbit, rat, or guinea pig, can be monitored (see e.g., Van

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Damme J. et al., *J. Exp. Med.*, 176: 59-65 (1992); Zachariae, C.O.C. et al., *J. Exp. Med.* 171: 2177-2182 (1990); Jose, P.J. et al., *J. Exp. Med.* 179: 881-887 (1994)). In one embodiment, skin biopsies are assessed  
5 histologically for infiltration of leukocytes (e.g., eosinophils, granulocytes). In another embodiment, labeled cells (e.g., stably transfected cells expressing a CKR-3 receptor, labeled with <sup>111</sup>In for example) capable of chemotaxis and extravasation are administered to the  
10 animal. Infiltration of cells in response to injection of a test sample (e.g., a compound to be tested in a suitable buffer or physiological carrier) is indicative of the presence of a ligand or promoter, such as an agonist, in the sample. These assays can also be modified to identify  
15 inhibitors of chemotaxis and leukocyte extravasation. For example, an inhibitor can be administered, either before, simultaneously with or after ligand or agonist is administered to the test animal. A decrease of the extent of infiltration in the presence of inhibitor as compared  
20 with the extent of infiltration in the absence of inhibitor is indicative of inhibition.

#### Diagnostic Applications

The present invention has a variety of diagnostic applications. These applications include, but are not  
25 necessarily limited to the applications discussed herein.

Mutation(s) in genes encoding a mammalian CKR-3 receptor protein can cause defects in at least one function of the encoded receptor, thereby reducing or enhancing receptor function. For instance, mutations which produce a  
30 variant of receptor or alter the level of expression, can reduce or enhance receptor function, reducing or enhancing, the inflammatory processes mediated by receptor.



For example, the methods of detecting or measuring receptor function can be used to characterize the activity of receptors in cells (e.g., leukocytes) of an individual or of receptors isolated from such cells. In these assays, 5 reduced or enhanced receptor function can be assessed.

The nucleic acids of the present invention provide reagents (e.g., probes, PCR primers) which can be used to screen for, characterize and/or isolate a defective mammalian CKR-3 receptor gene, which encodes a receptor 10 having reduced or enhanced activity. Standard methods of screening for a defective gene can be employed, for instance. A defective gene and the activity of the encoded receptor can be isolated and expressed in a suitable host cell for further assessment as described herein for 15 mammalian CKR-3 receptors. A number of human diseases are associated with defects in the function of a G-protein coupled receptor (Clapham, D.E., Cell, 75: 1237-1239 (1993); Lefkowitz, R.J., Nature, 365: 603-04 (1993)).

The antibodies of the present invention have 20 application in procedures in which receptor can be detected on the surface of cells. The receptor provides a marker of the leukocyte cell types in which it is expressed, particularly in eosinophils. For example, antibodies raised against a receptor protein or peptide can be used to 25 count cells expressing receptor. Cell counts can be used in the diagnosis of a variety of diseases or conditions in which increased or decreased leukocyte cell types (e.g., hypereosinophilia, for example in hypereosinophilic syndrome; hypoeosinophilia) are observed. The presence of 30 an increased level of eosinophils in a sample obtained from an individual can be indicative of eosinophil infiltration due to an inflammatory disease or condition, such as asthma, or an infection such as a parasitic infections. Alternatively, or in addition, the antibodies can be used 35 to sort cells which express receptor from among a mixture

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of cells. Suitable methods for counting and/or sorting cells can be used for this purpose (e.g., flow cytometry, fluorescence activated cell sorting).

- Furthermore, the antibodies can be used to detect or
- 5 measure decreased or increased expression of receptor in various diseases or conditions in which inflammatory processes of leukocytes are altered (e.g., increased or decreased relative to a suitable control, such as the level of expression in a normal individual). For example,
- 10 leukocytes (e.g., eosinophils, lymphocytes such as T lymphocytes, monocytes, basophils) can be obtained from an individual and a suitable immunological assay (e.g., ELISA, FACS analysis) can be used to assess the level of expression. The level of expression of a mammalian CKR-3
- 15 receptor can be used in the diagnosis of a disease or condition in which increased or decreased expression of a mammalian CKR-3 receptor is present.

#### Transgenic Animals

- Transgenic animals, in which the genome of the animal
- 20 host is altered using recombinant DNA techniques, can be constructed. In one embodiment, the alteration is not heritable (e.g., somatic cells, such as progenitor cells in bone marrow, are altered). In another embodiment, the alteration is heritable (the germ line is altered).
- 25 Transgenic animals can be constructed using standard techniques or other suitable methods (see e.g., Cooke. M.P. et al., *Cell*, 65: 281-291 (1991) regarding alteration of T lymphocytes; Hanahan, D., *Science*, 246: 1265-1275, (1989)).

- In one aspect, an endogenous mammalian CKR-3 receptor
- 30 gene can be inactivated or disabled, in whole or in part, in a suitable animal host (e.g., by gene disruption techniques) to produce a transgenic animal. Nucleic acids of the present invention can be used to assess successful construction of a host containing an inactivated or

disabled CKR-3 gene (e.g., by Southern hybridization). In addition, successful construction of a host containing an inactivated or disabled CKR-3 gene can be assessed by suitable assays which monitor the function of the encoded  
5 receptor.

In another embodiment, a nucleic acid encoding a mammalian CKR-3 receptor protein or polypeptide is introduced into a suitable host to produce a transgenic animal. In a preferred embodiment, endogenous CKR-3  
10 receptor genes present in the transgenic animals are inactivated (e.g., simultaneously with introduction of the nucleic acid by homologous recombination, which disrupts and replaces the endogenous gene). For example, a transgenic animal (e.g., a mouse, guinea pig, sheep)  
15 capable of expressing a nucleic acid encoding a mammalian CKR-3 receptor of a different mammalian species (e.g., a human) in leukocytes (such as eosinophils, lymphocytes (e.g., T lymphocytes) can be produced, and provides a convenient animal model for assessing the function of the  
20 introduced receptor. In addition, a compound can be administered to the transgenic animal, and the effect of the compound on an inflammatory process mediated by receptor can be monitored in a suitable assay ((see e.g., Weg, V.B. et al., *J. Exp. Med.*, 177: 561 (1993); Abraham,  
25 W.M. et al., *J. Clin. Invest.*, 93: 776 (1994)). In this manner, compounds which inhibit or promote receptor function can be identified or assessed for *in vivo* effect.

#### Methods of Therapy

Modulation of mammalian CKR-3 receptor function  
30 according to the present invention, through the inhibition or promotion of at least one function characteristic of a mammalian CKR-3 receptor, provides an effective and selective way of inhibiting or promoting leukocyte-mediated inflammatory action. One or more ligands, inhibitors

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and/or promoters of CKR-3 receptor function, such as those identified as described herein, can be used to modulate leukocyte function for therapeutic purposes.

As major eosinophil and lymphocyte chemokine receptors, mammalian CKR-3 receptors provide a target for interfering with or promoting eosinophil and/or lymphocyte function in a mammal, such as a human. Consistently, co-localization of T cells and eosinophils is observed in certain inflammatory infiltrates. Thus, compounds which inhibit or promote CKR-3 receptor function, such as ligands, inhibitors and promoters identified according to the present method, are particularly useful for modulating eosinophil and/or lymphocyte function for therapeutic purposes.

Thus, the present invention provides a method of inhibiting or promoting an inflammatory response in an individual in need of such therapy, comprising administering a compound which inhibits or promotes mammalian CKR-3 receptor function to an individual in need of such therapy.

In one embodiment, a compound which inhibits one or more functions of a mammalian CKR-3 receptor (e.g., a human CKR-3 receptor) is administered to inhibit (i.e., reduce or prevent) inflammation. As a result, one or more inflammatory processes, such as leukocyte emigration, chemotaxis, exocytosis (e.g., of enzymes, histamine) or inflammatory mediator release, is inhibited. For example, eosinophilic infiltration to inflammatory sites (e.g., in asthma) can be inhibited according to the present method.

In another embodiment, a compound which promotes one or more functions of a mammalian CKR-3 receptor (e.g., a human CKR-3 receptor) is administered to stimulate (induce or enhance) an inflammatory response, such as leukocyte emigration, chemotaxis, exocytosis (e.g., of enzymes, histamine) or inflammatory mediator release, resulting in

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the beneficial stimulation of inflammatory processes. For example, eosinophils can be recruited to combat parasitic infections.

In addition to primates, such as humans, a variety of other mammals can be treated according to the method of the present invention. For instance, mammals including, but not limited to, cows, sheep, goats, horses, dogs, cats, guinea pigs, rats or other bovine, ovine, equine, canine, feline, rodent or murine species can be treated. However, the method can also be practiced in other species, such as avian species (e.g., chickens).

Diseases and conditions associated with inflammation and infection can be treated using the method. In a preferred embodiment, the disease or condition is one in which the actions of eosinophils and/or lymphocytes are to be inhibited or promoted, in order to modulate the inflammatory response.

Diseases or conditions of humans or other species which can be treated with inhibitors of CKR-3 receptor function, include, but are not limited to:

- inflammatory or allergic diseases and conditions, including respiratory allergic diseases such as asthma, allergic rhinitis, hypersensitivity lung diseases, hypersensitivity pneumonitis, eosinophilic pneumonias (e.g., Loeffler's syndrome, chronic eosinophilic pneumonia), interstitial lung diseases (ILD) (e.g., idiopathic pulmonary fibrosis, or ILD associated with rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, systemic sclerosis, Sjogren's syndrome, polymyositis or dermatomyositis); systemic anaphylaxis or hypersensitivity responses, drug allergies (e.g., to penicillin, cephalosporins), insect sting allergies; inflammatory bowel diseases, such as Crohn's

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disease and ulcerative colitis; spondyloarthropathies;  
scleroderma; psoriasis and inflammatory dermatoses such as  
dermatitis, eczema, atopic dermatitis, allergic contact  
dermatitis, urticaria; vasculitis (e.g., necrotizing,  
5 cutaneous, and hypersensitivity vasculitis);

- eosinophilic myositis, eosinophilic fasciitis;

- autoimmune diseases, such as rheumatoid arthritis,  
psoriatic arthritis, multiple sclerosis, systemic lupus  
erythematosus, myasthenia gravis, juvenile onset diabetes,  
10 glomerulonephritis, autoimmune thyroiditis, Behcet's  
disease;

- graft rejection (e.g., in transplantation),  
including allograft rejection or graft-versus-host disease;

- cancers with leukocyte infiltration of the skin or  
15 organs;

- other diseases or conditions in which undesirable  
inflammatory responses are to be inhibited can be treated,  
including, but not limited to, reperfusion injury,  
atherosclerosis, certain hematologic malignancies,  
20 cytokine-induced toxicity (e.g., septic shock, endotoxic  
shock), polymyositis, dermatomyositis.

Diseases or conditions of humans or other species  
which can be treated with promoters of CKR-3 receptor  
function, include, but are not limited to:

- 25 • immunosuppression, such as that in individuals with  
immunodeficiency syndromes such as AIDS, individuals  
undergoing radiation therapy, chemotherapy, therapy for  
autoimmune disease or other drug therapy (e.g.,

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corticosteroid therapy), which causes immunosuppression; immunosuppression due congenital deficiency in receptor function or other causes;

- infectious diseases, such as parasitic diseases, including, but not limited to helminth infections, such as nematodes (round worms); (Trichuriasis, Enterobiasis, Ascariasis, Hookworm, Strongyloidiasis, Trichinosis, filariasis); trematodes (fluxes) (Schistosomiasis, Clonorchiasis), cestodes (tape worms) (Echinococcosis, Taeniasis saginata, Cysticercosis); visceral worms, visceral larva migrans (e.g., *Toxocara*), eosinophilic gastroenteritis (e.g., *Anisaki* spp., *Phocanema* ssp.), cutaneous larva migrans (*Ancylostoma braziliense*, *Ancylostoma caninum*).

15 *Eosinophils as the Target Cell in Certain Inflammatory Reactions, Particularly Asthma*

- Eosinophils are produced in the bone marrow and circulate to the tissues, predominantly to mucosal tissues, such as the lungs, gastrointestinal tract, and genitourinary tract. Eosinophils typically constitute 1-3% of leukocytes in the blood. However, in people suffering from allergic diseases and helminthic parasitic infections, increased eosinophil accumulation occurs in the tissues or the blood. Eosinophils accumulation can be both beneficial and detrimental to the host.

- For example, eosinophils possess numerous granules, containing cationic proteins. Degranulation of eosinophils, triggered, for example, by the engagement of IgG, IgA, or IgE receptors, or by stimulation by inflammatory mediators such as platelet-activating factor (PAF), leukotrienes, or chemokines, leads to release of the components in the granule. Products from eosinophils also cause damage to host cells. The most damaging are the cationic proteins,

which are detectable in elevated concentrations in patients with asthma. Eosinophils also generate a number of inflammatory mediators, including Leukotriene C4, and platelet-activating factor (PAF). These mediators contract  
5 airway smooth muscle, promote the secretion of mucus, alter vascular permeability, and elicit further eosinophil and neutrophil infiltration.

Eosinophils are involved in the initiation and maintenance of allergic/asthma diathesis. Thus, in a  
10 preferred embodiment, the method can be used to treat asthma or hypersensitivity (allergic) states, particularly those involving mucosal tissues, as well as in other eosinophil-associated diseases. In a particularly preferred embodiment, a compound which inhibits one or more  
15 function of a mammalian CKR-3 receptor (e.g., a human CKR-3 receptor) is administered to an individual with asthma.

Eosinophils are clearly important in the host defense against and destruction of, large, nonphagocytatable organisms, such as multicellular helminthic parasites.  
20 Eosinophils are also important effector cells in immune reactions against other pathogens that induce high levels of IgE antibodies. Accordingly, the method can be used to treat infectious diseases, such as parasitic diseases, to stimulate or promote inflammatory defenses, or to suppress  
25 inflammatory responses which are destructive to the host.

#### *Eosinophils and Asthma Pathogenesis*

Asthma is characterized by the obstruction of the airways or bronchi, and results from a bronchial hyperresponsiveness and rapid constriction in response to a  
30 wide range of pharmacological mediators. Chronic inflammation of the bronchial mucosal lining is widely believed to play a fundamental role in the development of asthma.



Intense infiltration of the bronchial mucosa with eosinophils, macrophages and lymphocytes is observed in asthma and other hypersensitivities. Often the selective migration of eosinophils to inflamed airways can be striking, and appears to result from the selective binding of eosinophils to endothelium and extraction from the blood. Eosinophils in particular are implicated as the causative agents of bronchial mucosal injury. Studies of asthmatic patients suggest that blood eosinophil counts correlate with the degree of bronchial hyperresponsiveness. In addition, bronchial biopsies and bronchoalveolar lavage fluid from asthmatics show a clear relationship between the degree of eosinophilia and clinical severity. Thus, there is a strong connection between the presence of eosinophils and adverse immune reactions, particularly in asthma.

A major chemokine receptor on eosinophils and lymphocytes, that functions in selective leukocyte chemotaxis, extravasation and activation in response to chemoattractant, provides an excellent target for interfering with eosinophil recruitment. For example, administration of an inhibitor of at least one function of a mammalian (e.g., human) CKR-3 receptor, such as by inhibiting chemokine binding thereto, can provide an effective and selective way of treating asthma. By reducing or preventing recruitment (extravasation, infiltration) of leukocytes, particularly eosinophils, to inflamed lung and airway tissues, and/or reducing leukocyte function in those tissues, the destructive inflammatory processes of asthma can be inhibited, and the symptoms alleviated.

There is evidence that the blockage of eosinophil recruitment to the lung can alleviate the symptoms of asthma. Administration of a monoclonal antibody reactive with  $\alpha 4$  integrin was reported to inhibit the accumulation of eosinophils into the lung and airways, and blocked the

airway hyperresponsiveness to antigen challenge in sheep. In a primate model of asthma, a monoclonal antibody to ICAM-1 is reported to attenuate airway eosinophilia and hyperresponsiveness. In addition, in a guinea pig model  
5 for passive cutaneous anaphylaxis, *in vitro* pretreatment of eosinophils with the anti- $\alpha 4$  monoclonal was reported to suppress eosinophil accumulation. (see Wegner, C.D. et al., *Science*, 247: 456 (1990); Weg, V.B. et al., *J. Exp. Med.*, 177: 561 (1993); and Abraham, W.M. et al., *J. Clin. Invest.*, 93: 776 (1994) regarding these models).

#### *Modes of Administration*

According to the method, one or more compounds can be administered to the host by an appropriate route, either alone or in combination with another drug. An effective  
15 amount of a compound (e.g., a receptor peptide which inhibits ligand binding, an antibody or antibody fragment) is administered. An effective amount is an amount sufficient to achieve the desired therapeutic effect, under the conditions of administration, such as an amount  
20 sufficient for inhibition or promotion of a CKR-3 receptor function, and thereby, inhibition or promotion, respectively, of an inflammatory response.

A variety of routes of administration are possible including, but not necessarily limited to oral, dietary,  
25 topical, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection), inhalation (e.g., intrabronchial, intranasal or oral inhalation, intranasal drops), routes of administration, depending on the disease or condition to be treated. For respiratory allergic  
30 diseases such as asthma, inhalation is a preferred mode of administration.

Formulation of a compound to be administered will vary according to the route of administration selected (e.g., solution, emulsion, capsule). An appropriate composition

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comprising the compound to be administered can be prepared in a physiologically acceptable vehicle or carrier. For solutions or emulsions, suitable carriers include, for example, aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles can include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles can include various additives, preservatives, or fluid, nutrient or electrolyte replenishers (See, generally, Remington's Pharmaceutical Science, 16th Edition, Mack, Ed. 1980). For inhalation, the compound can be solubilized and loaded into a suitable dispenser for administration (e.g., an atomizer, nebulizer or pressurized aerosol dispenser).

#### 15 EXEMPLIFICATION

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

#### EXAMPLE 1

#### 20 Chemotactic Properties of Human Eosinophils and an Eosinophilic-Like Cell Line

##### Chemotaxis of Human Eosinophils

To identify antagonists of eosinophilic chemokine receptor(s), it is necessary to identify the important chemokines for eosinophil chemotaxis, and determine the receptor(s) that these chemokines are binding to. Chemotaxis experiments were performed in a sensitive and improved chemotaxis assay, which employs an endothelial cell line grown on the polycarbonate membrane of the chemotaxis well.

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Isolation of Eosinophils

100 ml of heparinized blood was diluted 1:1 with PBS. 20 ml aliquots were layered over 65%, 75% Percoll step gradients. The gradients were centrifuged at 1500 rpm, 25 min at room temp. The eosinophil/neutrophil layers were transferred to a new tube and erythrocytes lysed by addition of 20 mls 0.2% NaCl for 1 min followed by the addition of 30 mls 1.8% NaCl. Cells were washed twice with a buffer consisting of PBS, 0.5% BSA, 0.5 mM EDTA. Cells were resuspended at  $5 \times 10^7$  cells/50  $\mu$ l in cold buffer (PBS, 0.5% BSA, 0.5 mM EDTA) and 50  $\mu$ l CD16 microbeads were added to the cells. The mixture was incubated at 4°C for 25 min followed by the addition of 900  $\mu$ l cold buffer. The miniMACS™ separation unit (Miltenyi Biotec, Inc., Auburn CA 95603) was used to deplete CD16 positive cells (neutrophils). Cells were loaded onto the column in 200  $\mu$ l aliquots. Flow-through cells were collected and assessed histologically. The eosinophil prep was > 99% pure.

Chemotaxis Assay

Chemokines were obtained from Peprotech, Inc. (Rocky Hill, N.J.). Chemotaxis experiments were performed using 3.0 micron Biocoat cell culture inserts (Collaborative Biomedical Products), in 24 well plates. Endothelial cells were grown to confluency on the inserts for two days prior to chemotaxis experiments. The endothelial cells used were a cell line termed ECV 304 (European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K.), which expresses endothelial cell markers such as von Willebrand factor, as well as ICAM-1 and VCAM-1. This endothelial cell line greatly facilitates these assays, since human umbilical vein endothelial cells can be variable in nature, can be used for only several passages, and grow much more slowly than ECV 304. The assay was conducted at 37°C for

1.5 hours, and migrated cells were counted using an inverted microscope.

### Results

The results, presented in Figure 4, are representative of at least five experiments. Growth of ECV 304 endothelial cells on the polycarbonate membrane reduced the background migration almost completely. Eosinophils applied to transendothelial chemotaxis assays showed migration to a number of chemokines, particularly RANTES, MCP-3, and to a lesser degree MCP-1. MIP-1 $\beta$ , IL-8, MCP-2, and IP-10 had little effect on eosinophil chemotaxis. MIP-1 $\alpha$  was chemotactic for eosinophils in some experiments, although generally was inactive. In these experiments, a range of chemokine concentrations was used, because of the variability in responsiveness of leukocytes to different chemokines, and uncertainties about the quality of chemokine preparations. A consistent finding was the high level of eosinophil chemotaxis to RANTES and MCP-3.

### Induction of RANTES Chemotaxis in Butyrate Differentiated HL-60 Cells

Eosinophils are difficult to isolate in sufficient quantities from human blood to be used for many studies, particularly high-throughput screening. Eosinophilic-like cell lines can provide a substitute for eosinophils. Some laboratories have found that HL-60 cells can differentiate down an eosinophilic pathway (Tagari, P. et al., *Int. Arch. Allergy Immunol.*, 101: 227-233 (1993); Van Riper, G. et al., *J. Immunol.*, 152: 4055-4061 (1994)). HL-60 cells were tested to determine whether such cells could emulate the chemotactic properties of eosinophils.

HL-60 cells were resuspended in RPMI (without HEPES) + 20% fetal calf serum (FCS) at  $0.5 \times 10^6$  cells/ml.

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n-Butyric acid (Sigma Chemical Co., St. Louis, MO; #B5887) was added to a final concentration of 0.4 mM from a stock solution of 1 M n-butyric acid. HL-60 cells were incubated at 37 °C, 5% CO<sub>2</sub> for four days. The eosinophilic-like nature of these HL-60 cells is indicated by the induction of RANTES and MCP-3 responsiveness in chemotaxis assays (Figures 5A-5B). Moreover ligand binding studies as well as northern blot analysis of chemokine receptor expression (see below) confirmed that chemokine receptors were induced on these differentiated HL-60 cells that are similar to those on eosinophils.

#### EXAMPLE 2

#### Identification of a Major Eosinophilic Chemokine Receptor

##### 15 Primer Selection and Design

Five chemokine receptor genes were aligned and compared to generate a set of degenerate oligonucleotides for use in PCR (Polymerase Chain Reaction) cloning of novel chemokine receptors from eosinophils. The selection of these five receptor genes was based on either the type of chemokine ligand with which they bind (Il-8 receptor A (IL8RA), Il-8 receptor B (IL8RB), MIP-1 $\alpha$  receptor (MIP1 $\alpha$ R)) or orphan receptors with significant sequence similarity to these receptors whose expression is reported to be restricted to lymphoid cells or tissue (Epstein Barr Inducible receptor-1 (EBI1R) and Burkitt's Lymphoma Receptor-1 (BLR1)). Receptor sequences were aligned by hand based on a number of published alignments (IL-8RA, Holmes et al., *Science*, 253: 1278-1280 (1991); IL-8RB, Murphy, P.A. et al., *Science*, 253: 1280-1283 (1991); MIP1 $\alpha$ /RANTES, Neote, K. et al., *Cell*, 72: 415-425 (1991); EBI1R, Birkenbach, M. et al., *J. Virol.*, 67: 2209-2220

(1993); and BLR1 (Dobner, T. et al., *Eur. J. Immunol.*, 22: 2795-2799 (1992)).

Sequences within transmembrane (TM) regions 2, 6 and 7 as well as a region just C-terminal to TM3 were selected as  
5 targets for degenerate oligonucleotide design based on the high degree of sequence similarity. The nucleotide sequences of the degenerate oligonucleotide primers are illustrated in the Table below.

**TABLE**

**Primer Set 2**

SEQ ID NO:

TM2a

7 Primer 2a-1 (forward) 5'- TAC CTG CTS AAC CTG GCC ITG GCI G  
8 Nested primer 2a-2 (forward) 5'- AC CTG GCC ITG GCI GAC CTM CTC TT

TM3

9 Primer 3F (forward) 5'- GAC CGY TAC CTG GCC ATI GTC CAY GCC  
10 Primer 3R (reverse) CTG GCR ATG GAC CGG TAI CAG GTR CGG - 5'

TM6b

11 Primer 6b-1 (reverse) GAR AMR ACC IRI GGG ATG TTR IAC CAI - 5'  
12 Nested primer 6b-2 (reverse) AAG RAI GAR GAR AMR ACC IRI GGG ATG T - 5'

TM7

13 Primer 7-1 (reverse) ACG SAG TTG GGI IAS IAG ATG CGG AAG - 5'  
14 Nested primer 7-2 (reverse) GTG WCG ACG SAG TTG GGI IAS IAG A - 5'

Nucleotide Abbreviations:

K = G/T  
M = A/C  
R = A/G  
S = C/G  
W = A/T  
Y = C/T



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Eosinophil Isolation and Purification

100 ml of heparinated blood was diluted 1:1 with PBS. 20 ml aliquots were layered over 65%, 75% Percoll step gradients. The gradients were centrifuged at 1500 rpm, 25 min at room temperature. The eosinophil/neutrophil layers were transferred to a new tube and erythrocytes lysed by addition of 20 mls 0.2% NaCl for 1 minute followed by the addition of 30 mls 1.8% NaCl. Cells were washed twice with a solution of phosphate buffered saline (PBS), 0.5% Bovine Serum Albumin (BSA), 0.5 mM ethylenediaminetetraacetic acid (EDTA). Cells were resuspended at  $5 \times 10^7$  cells/50  $\mu$ l in cold buffer (PBS, BSA, EDTA solution), and 50  $\mu$ l CD16 microbeads were added to the cells. The mixture was incubated at 4°C for 25 min followed by the addition of 900  $\mu$ l cold buffer. The miniMACS™ separation unit (Miltenyi Biotec, Inc., Auburn, CA 95603) was used to deplete CD16 positive cells (neutrophils). Cells were loaded onto the column in 200  $\mu$ l aliquots. Flow-through cells were collected and assessed histologically. By this criteria, the eosinophil prep was > 99% pure.

mRNA isolation and PCR

mRNA for RT-PCR (Reverse transcription-polymerase chain reaction) was extracted directly from purified cells using the Micro-FastTrack™ mRNA isolation kit purchased from Invitrogen. Quality of the mRNA was evaluated by PCR amplification of  $\beta$ -actin and/or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA prior to use with 7TMS degenerate primers.

20-50 ng of mRNA was reverse transcribed using a GeneAmp® RNA PCR kit (Perkin-Elmer) with oligo dT and/or random hexamers as primers in a 20  $\mu$ l final volume as specified by the manufacturer. 2-5  $\mu$ l of this cDNA (reverse transcribed eosinophil message) was mixed with 200

5  $\mu$ M dNTPs and 50-100 pmol of degenerate primers in a 50  $\mu$ l volume. Magnesium concentration and pH were optimized for each primer pair. The magnesium concentration ranged from 1.0 to 3.0 mM and pH ranged from 8.5 to 10.0. Although various cycle parameters were also evaluated, the conditions generally used were similar to the following: 3 cycles: 94°C, 30 sec; 37°C, 30 sec; 2 min ramp to 72°C, 1 min, followed by 30 cycles: 94°C, 45 sec; 48°C, 1 min; 72°C, 1 min. (ramp = gradual increase).

10 With regard to the 201 bp fragment isolated (see below), primer pairs 2a-1 and 7-1, or primer pairs 2a-1 and 3R, were used in a PCR reaction (as described above) in 60 mM Tris-HCl, pH 9.5 and 1.5 mM MgCl<sub>2</sub>. One  $\mu$ l of product from each reaction was used in a separate (second) round of  
15 PCR with "nested" primers 2a-2 and 3R. ("Nested" primers are primers which hybridize to sequences within the outside primers.) Reaction conditions for the nested PCR were exactly as described for the first PCR.

PCR products were assessed and separated by agarose  
20 gel electrophoresis, and appropriately sized fragments were purified and subcloned using the pCR-Script™ SK+ cloning kit (Stratagene). (Appropriate fragment sizes are as follows: for PCR with primer pairs from regions 2a and 7 (see Table above), ~700 bp; for PCR with primers from  
25 region 2a and primer 3R, ~200 bp; for PCR with primer 3F and primers from region 6b, ~400 bp, and for PCR with primer 3F and region 7 primers, ~550 bp.) Expected fragment sizes were predicted based upon the hypothesis that a related receptor protein would share some structural  
30 similarity.

#### Rapid Screening Assay

In order to screen a large number of clones quickly for novel members of the 7TMS family, the inserts of

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bacterial colonies obtained as described above (i.e., transformants of plasmids comprising appropriately sized fragments subcloned into pCR-Script SK+), were screened by PCR using T3 and KS primers complementary to the sequence flanking the polylinker of pCR-Script™. In particular, a portion of a bacterial colony from an overnight transformation was mixed directly with 40 µl of a PCR mixture containing 200 µM dNTPs, 20 mM Tris, pH 8.5, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 50 pmol each primers and 0.25 units Taq polymerase. Cycle conditions were 25 cycles: 94°C, 20 sec; 55°C, 20 sec; 72°C, 30 sec. Inserts of the correct size were identified by evaluating 20 µl of PCR product on 1.5% agarose gels. The remaining 20 µl of the reaction was digested with Alu I, Hha I, and Rsa I (triple digestion) and resolved on a 12% polyacrylamide gel to screen for different digestion patterns. Clones of different patterns were then selected for sequence analysis.

### Results

Sequence analysis of PCR fragment, generated from degenerate oligos, identified a 201 bp partial cDNA clone in pCR-Script. (The degenerate oligos were 2a-1, 2a-2, 3F, 3R and 7-1). This partial clone, designated Eos L2 (also referred to as L2 and EL2), was found to have 78.3% amino acid similarity (81.1% nucleic acid similarity) to the MIP1α/RANTES receptor and 60.8% amino acid similarity (61.6% nucleic acid similarity) to the MCP-1 receptor. A search of the most current sequence data bases revealed this partial clone to be unique.

### Southern and Northern Analysis

The PCR fragment was labeled and used to probe both Southern and Northern blots. To prepare the PCR probe, the 201 bp fragment was released from the pCR-Script vector

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with restriction enzymes EcoRI and Not I. This digested resulted in a fragment of 240 bp comprised of the 201 bp fragment plus 39 base pairs of polylinker from the vector. The fragment was separated from vector by electrophoresis through agarose gel, and purified by (Magic Mini Prep, Promega Corp. Madison, WI) exactly as recommended by the manufacturer. Approximately 200 ng of material was labeled with the Random Primed DNA Labeling Kit purchased from Boehringer Mannheim following the manufacturer's recommended labeling protocol.

For Southern blots, genomic DNA (purchased from Clontech Laboratories, Inc., Palo Alto, CA) was digested with restriction enzyme overnight and separated by electrophoresis on a 0.7% agarose gel followed by capillary transfer to Hybond-N nylon membrane (Amersham). Hybridization was in 6x SSC (1x SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) containing 5x Denhardt's solution (1x Denhardt's solution is 0.02% bovine serum albumin, 0.02% ficoll, 0.02% polyvinylpyrrolidone), 10% w/v dextran sulfate, 2% SDS, and sheared salmon sperm DNA (100 µg/ml) overnight at 65°C. The membrane was rinsed twice in 2X SSC, 0.5% SDS at 65°C followed by two washes (15 min each) in 0.2X SSC, 0.5% SDS at 65°C.

The Southern hybridization revealed a single strongly hybridizing fragment and a single weakly hybridizing fragment with each enzyme used. The weakly hybridizing fragment is likely to be the MIP1α1/RANTES receptor.

Multiple Tissue Northern Blots were purchased from Clontech Laboratories, Inc., Palo Alto, CA). ExpressHyb™ Solution was also purchased from Clontech Laboratories, Inc. The Multiple Tissue Northern Blots were carried out as recommended by the manufacturer. The probe was as described above for Southern blots. The results of the Northern hybridization showed high levels of a - 1.6 kb

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message in spleen, peripheral blood leukocytes and thymus. Additional Northern analyses are presented in Example 5.

#### Genomic Library Screening

A human genomic phage library constructed in the EMBL3 SP6/T7 vector, purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA), was screened with the 201 bp PCR fragment to obtain a full-length clone. Approximately 25,000 plaque forming units were mixed with 600  $\mu$ l of an overnight bacterial culture of *E. coli* strain K802 provided with the library in NZCYM top agarose and plated on 150 mm petri dishes containing NZCYM agar (NZCYM broth, Agar and Agarose were purchased from Gibco/BRL). After incubation at 37°C for 7 hours, the plates were overlaid with BA-85 nitrocellulose membranes (Schleicher and Schuell, Keene, NH) for 5 minutes to allow transfer of phage to membrane. The membranes were then soaked for 5 minutes in Denturing Solution (1.5 M sodium chloride, 0.5 N sodium hydroxide) followed by neutralization in 1.5 M sodium chloride, 0.5 M Tris, pH 8.0. The filters were allowed to air dry for 15 minutes and then baked for two hours at 80°C under vacuum. The filters were then hybridized as described above for the Southern Blot. The 201 bp PCR fragment contained the nucleotides between oligonucleotide primers 2a-2 (TM2) and 3R (TM3).

One genomic phage clone, designated Eos L2.8, contained an insert which comprises the 1.8 kb Hind III fragment seen on Southern blots (complete insert size was not determined, but is ~17 kb).

Phage clone Eos L2.8 was digested with Hind III restriction enzyme and electrophoresed on an agarose gel. A Hind III fragment of approximately 1.8 kb was cut out, electroeluted from agarose, phenol/chloroform extracted and precipitated with ethanol. The 1.8 kb fragment was resuspended in water and ligated into the Hind III site of

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the pBluescript II KS+ vector (Stratagene) followed by transformation into DH5 $\alpha$  competent cells purchased from Gibco/BRL.

Both strands of this Hind III fragment were  
5 sequenced, and the fragment was found to contain the entire amino acid coding region for the Eos L2 receptor (a human CKR-3 receptor). Comparison of this sequence and the cDNA clone described below indicates that the clone is a full-length clone. The open reading frame of 1065 nucleotides  
10 encodes a protein of 355 amino acids (SEQ ID NO:2) with a predicted molecular mass of 41 Kd.

Comparison of the sequence of the full-length Eos L2 receptor with MIP1 $\alpha$ /RANTES and MCP-1 receptors revealed a 73.4% and 60.5% amino acid similarity, respectively. For  
15 this comparison, sequences were aligned by hand and the number of similar amino acids, divided by the total number of amino acids was multiplied by 100.)

The sequences were also aligned by the Clustal method using MegAlign<sup>TM</sup> (DNASTAR, Inc.). Comparison with other  
20 chemokine receptor sequences revealed a 62%, 47%, and 41% amino acid sequence similarity to CKR-1, CKR-2B, and CKR-4, respectively. In contrast, the amino acid sequence similarity to IL-8 receptors A and B was only 27% for both receptors. The sequence similarity of this receptor to  
25 MIP1 $\alpha$ /RANTES and MCP-1 receptors, both C-C chemokine receptors, is consistent with the results reported herein which indicate that Eos L2 is a C-C chemokine receptor.

### EXAMPLE 3

#### Expression of Eos L2 in Transfected Cell Lines

##### 30 FLAG-tagged Eos L2 (CKR-3) Receptor construct

An Eos L2 receptor fusion protein was constructed as follows:

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1. A FLAG-PAP receptor construct in pCDM8 (constructed as reported in Kunz, D. et al., *J. Biol. Chem.*, 267: 9101-9106 (1992)) was double digested with Hind III and EcoRI to release a 48 bp fragment containing  
5 nucleotides which encode the FLAG peptide. The nucleotide sequence is AAGCTTCCA GCA GCC ATG GAC TAC AAG GAC GAC GAT GAC AAA GAATTC (SEQ ID NO:15). The amino acid sequence is MDYKDDDDKEF (SEQ ID NO:16). The 48 bp Hind III/EcoRI fragment containing the FLAG nucleotides subcloned into the  
10 HindIII/EcoRI sites of the pcDNA3 vector (Invitrogen, San Diego, CA) giving rise to pcDNA3/FLAG.

2. The pBluescript II KS+ vector containing the 1.8 kb Eos L2 Hind III fragment was digested with BamHI and Xho I to release a 1.261 kb fragment. This BamHI-XhoI  
15 fragment contains nucleotides encoding Eos L2 amino acids 91 through the stop codon plus the same 3' untranslated region and 21 bp of pBluescript II KS+ vector.

3. Two PCR primers were generated to amplify the 5' end of the Eos L2 gene, but removing the first Met  
20 and engineering in an EcoRI site which will be compatible with the EcoRI site described above in step 1. The 5' primer (SEQ ID NO:17) was:

EcoRI  
5'-TTAA GAATTC ACA ACC TCA CTA GAT AC  
25 This primer contains an EcoRI site and the first 17 nucleotides of the EosL2 gene except for the Met codon.

The 3' primer (SEQ ID NO:18) was:

BamHI  
30 5'-CATAGT GGATCC AGAATG  
This primer primes in the Eos L2 gene just 3' to the BamHI site. Amplification with these two primers using the pBluescript II KS+ vector containing the 1.8 kb Eos L2 fragment as template will amplify a 280 bp fragment  
35 containing the 5' end of the Eos L2 which can be digested

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with EcoRI and BamHI to give a fragment for ligation as described below.

Conditions for amplification were: 100 ng of pBluescript II KS+ containing the 1.8kb EosL2 fragment was  
5 combined with 200  $\mu$ M dNTPs and 50 pmol of primers in a 50  $\mu$ l reaction volume. The final magnesium concentration was 2.5  $\mu$ M and the pH was 8.0. The fragment was amplified with 25 cycles of 94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec. The amplified product was separated on agarose gel and purified  
10 by electroelution as described above. The fragment was digested with EcoRI and BamHI purified again on agarose gel.

4. For construction of the Flag-tagged EosL2 gene, the pcDNA3 vector containing the FLAG fragment  
15 (described in step 1) was digested with EcoRI and Xho I. The vector fragment (an EcoRI-XhoI fragment comprising the FLAG coding sequence) was separated from the polylinker fragment by electrophoresis, and the vector fragment was purified as described for other electroeluted fragments.  
20 The vector fragment was combined with the EcoRI-BamHI fragment generated by PCR in step three. These two fragments were combined with the 1.261 kb BamHI-XhoI fragment from step two. All three fragments were triple ligated together to yield the FLAG-tagged Eos L2 receptor  
25 in pcDNA3. Ligated DNA was transformed into DH5 $\alpha$ .

#### Transient Transfectants

293 cells (ATCC Accession No. CRL 1573) were grown in Minimal Essential Medium (MEM) Alpha Medium obtained from Gibco/BRL and supplemented with 10% fetal Calf Serum,  
30 Glutamine, and Penicillin/Streptomycin (all from Gibco/BRL). For each transient transfection,  $2 \times 10^6$  293 cells were plated 1 day before transfection in a 35-mm tissue culture dish. On the day of transfection, the cells



(which grow attached to the dish) were washed 1 x with Phosphate Buffered Saline (PBS, Gibco/BRL) and a mixture of DNA and lipofectAMINE™ Reagent (Gibco/BRL) were applied to the cells.

- 5        The DNA/lipofectAMINE™ reagent mixture was made by incubating 2 µg of Flag-tagged Eos L2 receptor expression vector in a final volume of 100 µl OptiMEM™ (Gibco/BRL) with 12 µl of LipofectAMINE™ reagent in a 100 µl volume for 45 minutes at room temp. The final mixture volume is 200  
10 µl. After the 45 minute incubation, 800 µl of OptiMEM™ is added to the 200 µl of DNA/lipofectAMINE™ reagent and the 1 ml of solution is layered over the cells as described above. The cells were then incubated at 37°C for 5 hours at which time 1 ml of MEM Alpha Medium supplemented as  
15 described above is added. The cells are incubated for an additional 12 hours at which time all medium is removed and the cells washed 2 x with PBS and 2 mls of MEM Alpha medium supplemented as described above is added. The transfected cells are then incubated for an additional 72 hours. The  
20 cells are harvested by gently pipetting them after incubation in PBS 10 mM EDTA.

- Cell surface expression of a FLAG-tagged Eos L2 receptor was demonstrated in the transiently transfected 293 cells. Approximately 2.6% of the cells express the  
25 receptor on the surface as determined by immunofluorescent staining and FACS analysis. Levels of expression in some cells were found to be as much as 2 logs greater than background indicating that high levels of expression can be achieved in this cell line. As the Eos L2 gene is carried  
30 by the pcDNA3 expression vector (Invitrogen Corp., San Diego, CA), which contains the neomycin resistance gene, stable 293 transfectants can be selected using geneticin (G418) selection.

### Stable Cell Lines

Over 500 stable lines of mouse L1-2 pre-B cells have been generated with the FLAG-tagged receptor. L1-2 pre-B cells were obtained from (Dr. Eugene Butcher, Stanford University, Stanford, CA), and were maintained in RPMI-1640 (Gibco/BRL), supplemented with 10% bovine serum albumin, and Pen/Strep, sodium pyruvate and  $\beta$ -mercaptoethanol. Cells from over 200 clones were screened for surface expression by staining with M2 anti-FLAG monoclonal antibody (International Biotechnologies, Inc., New Haven, CT), followed by anti-mouse Ig-FITC (Jackson ImmunoResearch Laboratories, Inc.), and analyzed by fluorescence activated cell sorting (FACS). Immunofluorescent staining and FACS analysis was performed as described in Current Protocols in Immunology, Vol. 1, Coligan, J. et al., Eds., (John Wiley & Sons, Inc.; New York, NY). Results of the FACS analysis for several cell lines revealed a number of clones which express high levels of the Eos L2 flagged receptor (Figure 6). Untransfected cells (not shown) were negative for staining. Stable cell lines with high level expression can be used as immunogens for the production of antibodies reactive with the Eos L2 receptor. In addition, these cell lines are useful for studying chemotaxis and ligand binding.

### 25 Baculovirus Expression

For construction of a baculovirus expression vector, the Flag-tagged Eos L2 receptor in pcDNA 3 was digested with HindIII to remove the Flag-tagged gene. The HindIII fragment containing the gene was blunt ended by filling in the overhangs with Klenow fragment and dNTP's. The blunt ended fragment was subcloned into the Sma I site of pVL1393 (Invitrogen). 2.0  $\mu$ g of the pVL1393 vector containing the Eos L2 gene was mixed with 0.5  $\mu$ g of AcMNPV viral DNA (Invitrogen) and co-transfected into Sf9 insect cells

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(Invitrogen) with Insectin™ (Invitrogen) according to the manufacturer's instructions. The SF-900 media (serum free) was replaced with 5 ml of SF-9 culture medium (Grace's Supplemented Insect Media (Gibco/BRL) containing 10% fetal calf serum) on the following day, and the cells were allowed to grow for five days. Recombinant virus was plaque purified as described in D.R. O'Reilly, L.K. Miller, and V.A. Luckow (1994) *Baculovirus expression vectors: A Laboratory Manual*, Oxford University Press, pp. 149-158.

10 Expression of the Eos L2 receptor was obtained on Sf9 cells by infecting Sf9 cells with the plaque purified recombinant virus described above. The Sf9 cells ( $2 \times 10^6$  cells/ml) were infected at a multiplicity of infection of 10:1. The infection proceeded for 72 hours at which time the cells were stained with the M2 anti-FLAG antibody.

15 Successful expression of this receptor was also achieved with a baculovirus expression system in Sf9 cells. Good levels of expression have been achieved based on staining with anti-FLAG antibody (see Example 5). Ligand binding was also achieved with the same cells Sf9 transfectants shown by FACS to be expressing receptor. While definitive cell surface expression was shown by propidium iodide exclusion, expression on these cells appeared to be low, as compared with a negative control (i.e., Sf9 cells transfected with expression vector lacking the Eos L2 gene insert). Length of infection can be decreased, and MOI can be further optimized, for higher cell surface expression.

EXAMPLE 4Ligand Binding StudiesLigand Binding Procedure

Cells transfected with Eos L2 receptors or normal  
5 human eosinophils purified (see above) were washed in Hanks  
Balanced Saline Solution (HBSS), then resuspended in  
binding buffer: 50 mM HEPES, 1 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , 0.5%  
Bovine Serum Albumin (BSA), pH 7.3. In microfuge tubes,  $5 \times 10^5$   
10 cells were incubated with 0.1 nM radiolabeled  
chemokine (purchased from New England Nuclear,  
Massachusetts) in 200  $\mu\text{l}$  aliquots at room temperature for  
60 minutes. The cells were either incubated with  
radiolabeled chemokine alone, or together with unlabeled  
chemokines (from PeproTech) as competitors, which were used  
15 at the indicated concentrations. At the end of incubation,  
cells were washed 3 times in the binding buffer, each wash  
consisting of centrifugation in a microfuge at  $7,000 \times g$   
for 2 minutes. After the wash, the pellets were  
transferred into LP3 tubes and the radioactivity of the  
20 cells, which represented the amount of binding was measured  
in a gamma counter. All samples were in duplicates and all  
the experiments were repeated at least 3 times. Scatchard  
Plot was calculated from the binding data by MicroSoft  
Excell and CricketGraph on a Macintosh computer.

25 Binding to Human Eosinophils

Based on the findings from chemotaxis assays (see  
Example 1), the ligand binding studies focused on RANTES,  
MIP-1 $\alpha$  and MCP-3. The ligand binding studies were carried  
out using radiolabeled chemokines and various 'cold'  
30 chemokines as competitors. Purified normal human  
eosinophils were incubated with either 0.1 nM  $^{125}\text{I}$ -labeled  
MIP-1 $\alpha$  or RANTES in the presence or absence of various cold

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chemokines (250 nM MIP-1 $\alpha$ , RANTES, IL-8, MCP-1 or MCP-3). After extensively washing the cells, the binding was measured by a gamma counter.

Figure 7 is a histogram illustrating the binding of human eosinophils to RANTES and MIP-1 $\alpha$ . These results suggest that eosinophils bind only weakly to MIP-1 $\alpha$ , and that this binding can be inhibited by MIP-1 $\alpha$  itself and by other  $\delta$ -family chemokines, e.g., MCP-1, MCP-3 and RANTES (Figure 7). In contrast, eosinophils bound RANTES more abundantly (Figure 7). Binding by RANTES could not be inhibited efficiently by excess amount of 'cold' MIP-1 $\alpha$  (Figure 8), suggesting that on eosinophils, there could be distinguished receptors for MIP-1 $\alpha$  and RANTES.

Scatchard plot analysis revealed that there are  $1.8 \times 10^3$  MIP-1 $\alpha$  binding sites with an affinity of 91 pM. The analysis also revealed a lower affinity (883 pM) receptor for RANTES, having more binding sites ( $3.6 \times 10^4$ /cell). Under the conditions used, there was no significant MCP-1 binding to eosinophils (not shown), and MCP-1 did not inhibit RANTES binding except at very high concentrations (2500-fold excess, Figure 8).

#### Binding to Butyric Acid-Differentiated HL-60 Cells

Butyric acid-differentiated HL-60 cells were used in the ligand binding assay to determine whether these cell behave in the same way as eosinophils. Assays were performed as described above, using  $0.5 \times 10^6$  HL-60 cells incubated with 0.1 nM  $^{125}$ I-labeled MIP-1 $\alpha$  or RANTES in the absence or presence of 250 nM cold chemokine (MIP-1 $\alpha$ , RANTES, MCP-3, IL-8, MCP-1). In these cells, both MIP-1 $\alpha$  and RANTES bound equally well and both could cross-inhibit each other (Figure 9). This observation is consistent with the chemotaxis assays (see Example 1) in which both chemokines induced transendothelial migration of

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eosinophils (Figure 8). It appears that during the induction process, both receptors were unregulated since the binding of MIP-1 $\alpha$  and RANTES was insignificant in undifferentiated HL-60 cells (data not shown).

#### 5 Eos L2 Receptor Transfectants

Following the cloning and expression of the Eos L2 receptor, transfected cells were used to test binding to a number of chemokines. The first attempts using 293 transfectants were unsuccessful, as the addition of cold  
10 chemokines interfered with binding, a phenomenon observed by other investigators. In contrast, using baculovirus infected SF9 cells, good RANTES binding could be detected (Figure 10). The assay conditions for SF9 cells were different from that of mammalian cells. Binding of 0.1 nM  
15 <sup>125</sup>I-labeled RANTES took place in 50 mM HEPES, pH 7.3, 5 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>, supplemented with 0.5% BSA. After 60 minutes at room temperature, the cells were washed three times in the binding buffer containing 0.5 M NaCl, and the radioactivity in the cell pellets was counted using a gamma  
20 counter.

In these ligand binding assays, the most effective heterologous competitor of MIP-1 $\alpha$  or RANTES binding was MCP-3. In fact, MCP-3 also effectively inhibited MCP-1 binding to activated T cells. Thus, MCP-3 appears to bind  
25 to CKR-1, CKR-2 and CKR-3 (CKR-1, Gao, J.L., et al., *J. Exp. Med.*, 177: 1421-1427 (1993) and Neote, K., et al., *Cell*, 72: 415-425 (1993); CKR-2, Charo, I.F., et al., *Proc. Natl. Acad. Sci. USA*, 91: 2752-2756 (1994) and Myers, S.J., et al., *J. Biol. Chem.*, 270: 5786-5792 (1995)).

30 Radiolabeled MCP-3 (Peprotech, Inc. Rocky Hill, N.J.) was also used for binding studies. Figures 11A-11B are graphs illustrating the binding of MCP-3 to differentiated HL-60 cells. MCP-3 binding was carried out as described

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above with the following modifications. Cells were incubated with 0.1 nM  $^{125}\text{I}$ -labeled MCP-3. The binding buffer used was HBSS plus 0.5% BSA and 0.1% sodium azide. Binding took place at 37°C for 30 min. The unbound isotope was separated by spinning cells through 800  $\mu\text{l}$  of 20% sucrose, at 12,000 x g for 2 min. The tubes were then snap-frozen in dry ice, the tips cut off with a pair of pliers and counted.

#### EXAMPLE 5

##### 10 Expression of the Eosinophilic Chemokine Receptor

To confirm that the Eos L2 receptor is the functional receptor on eosinophils, the expression of the receptor was assessed by (a) Northern blot analyses, and (b) flow cytometry using monoclonal antibodies anti-peptide antibodies reactive with the receptor.

##### Purification of Human Eosinophils, Neutrophils, and PBMC

Eosinophils were isolated from heparinized blood of individuals with high levels of circulating blood eosinophils (5-17%) by combined density gradient centrifugation and negative selection with anti-CD16 magnetic beads (Hansel, T.T. et al., *J. Immunol. Meth.*, 122: 97 (1989)). Briefly, the granulocyte fraction from the Percoll centrifugation was incubated with CD16 microbeads (Miltenyi Biotec, Inc., Sunnyvale, CA) for 30 minutes. Cells were then passed through a MACS column (Miltenyi Biotec, Inc.), and eosinophils were collected in the flow-through. Eosinophils were shown histologically to be > 99% pure as determined by analysis of Diff-Quick-stained cytocentrifugation preparations by light microscopy.

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Human neutrophils were isolated from heparinized venous blood by Percoll density gradient centrifugation ( $d = 1.088$ ) at room temperature (Coligan et al., Eds., 1992, *Current Protocols in Immunology*, (John Wiley & Sons: New York, NY)). RBCs were removed by hypotonic lysis.

PBMCs were also isolated as described (Coligan et al., Eds., 1992, *Current Protocols in Immunology*, (John Wiley & Sons: New York, NY)). Monocytes were purified by CD14 positive selection with magnetic beads and T cells were purified by passage of lymphocytes over nylon wool. To generate CD3 blasts,  $2 \times 10^6$  PBMCs/ml in RPMI-1640 plus 10% FCS were added to tissue culture plates first coated with the anti-CD3 antibody TR77. After 4-6 days blasts were removed to fresh media and supplemented with IL-2 (Genzyme) at 50 units/ml.

Northern Analyses: CKR-3 Is Expressed Selectively in Eosinophils

Although eotaxin is a selective chemoattractant for eosinophils, the CKR-3 receptor also binds RANTES and MCP-3, which are known to attract monocytes and T cells. Message expression of the receptor was examined in various leukocyte populations.

The results of initial Northern hybridization (see Example 2) showed expression of a ~ 1.6 kb message in spleen, peripheral blood leukocytes, and thymus, and a number of leukocyte subpopulations, such as eosinophils and T cells, as well as in the HL-60 cell line. Message levels increased dramatically in the HL-60 cell line upon butyric acid induction down the eosinophilic pathway.

This message is likely to be that of Eos L2, since the message for the MIP1 $\alpha$ /RANTES receptor which cross-hybridizes on Southern blots is weak and is reported to be approximately 3.0 kb. When the original 201 bp PCR



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fragment is used as a probe in Southern blots, a strongly hybridizing 1.8 kb HindIII fragment is seen. This is the fragment that was cloned and discussed here. In addition to this fragment, a very weakly hybridizing fragment at about 10 kb is observed. This 10 kb fragment corresponds to the reported HindIII fragment size of the MIPl $\alpha$ /RANTES receptor. This MIPl $\alpha$ /RANTES receptor produces a message of approximately 3 kb which is not observed on Northern blots. Therefore, the ~1.6 kb message seen on Northern blots probably derives from Eos L2 gene. By far the most abundant expression of Eos L2 was observed in a preparation of purified eosinophils from a patient with hyper-eosinophilic syndrome (see Example 8).

Because of the high sequence similarity of CKR-3 to other CC chemokine receptors and the fact that the full-length clone hybridizes to multiple sequences in Southern blots, additional Northern analyses used a 250 bp fragment from the 3'-untranslated region of the genomic clone which does not cross-hybridize with other sequences in Southern blots. For hybridization, a 3'-untranslated region probe specific for CKR-3 was used encompassing nucleotides 1203-1453 (Figure 1C).

A Northern blot panel was prepared using RNA from different leukocyte populations, including monocytes, neutrophils, lymphocytes, T cells, T cell blasts produced by activation with CD3 MAb, and eosinophils. RNA was isolated using Trizol<sup>TM</sup> reagent (Gibco/BRL) following the manufacturer's recommended protocol. 15  $\mu$ g of total RNA isolated from each highly purified leukocyte population was separated on 1.2% formaldehyde agarose gels and transferred to Nytran-Plus<sup>TM</sup> nylon membrane (Schleicher and Schuell) and cross-linked using a Stratalinker<sup>®</sup>. Hybridization with radiolabeled 3'-untranslated region probe was with ExpressHyb<sup>TM</sup> Solution (Clontech) using the manufacturer's

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suggested protocol. Northern blots were exposed to X-OMAT AR film for 3-5 days with intensifying screen. CKR-3 specific probe was removed by boiling in 0.5% SDS and the blot re-probed with  $\beta$ -actin to control for variation in loading.

The only cell population which gave a detectable signal was eosinophils, where a message 1.8 kb in size was found. These results are consistent with the pattern of surface expression detected immunologically in Figures 13A-13D. Although message was not detected in resting or activated T cells in this experiment, it is possible that a subset of T cells may express the receptor.

Monoclonal Antibodies (MAbs) Reactive with the Eosinophilic Chemokine Receptor

MAbs reactive with the Eos L2 receptor were generated by immunizing mice with a synthetic peptide corresponding to the N-terminal 35 amino acids. The N-terminal 35 amino acids of Eos L2, deduced from the nucleotide sequence (see Figures 1A-1C; see also, SEQ ID NO:2), were synthesized and coupled to the carrier protein PPD (Purified Protein Derivative of *Mycobacterium tuberculosis*; Severn Biotech Ltd., Cambridge, U.K.).

Female Balb/C mice were immunized with 50  $\mu$ g of this peptide-peptide-carrier conjugate in PBS 4 times at 2 week intervals. Mice were injected intra-peritoneally with the peptide conjugate, using Freund's complete (first injection) and incomplete adjuvant (subsequent injections). The final immunization was injected intravenously without adjuvant. Polyclonal antiserum was also collected from mice immunized with synthetic peptide.

Two successful fusions were performed which generated over 15,000 hybridomas. Four days after the final injection, the spleen was removed and a single cell suspension prepared in serum free DMEM media. These cells

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were fused with the hybridoma fusion partner SP2/0, according to Galfre, G. et al. (Galfre, G. et al., *Nature*, 266: 550-552 (1977)). 20 ml of spleen cells and 20 ml of SP2/0 were combined, spun at 800g for 5 min and the media removed. A solution of 50% Polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN) prewarmed to 37°C was added to the cell pellet over 2 min, followed by 10 ml of DMEM media over 3 min. The cell suspension was spun at 400g for 3 min and the supernatant removed. The pellet was resuspended gently in DMEM media containing 20% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and HAT selection media (Boehringer Mannheim, Indianapolis, IN). Cells were plated into 96 well flat bottom microtiter plates at 200 µl/well. 10-14 days later, supernatants from the wells were screened for reactivity against the peptide using an enzyme-labeled anti-mouse antibody (Horseradish peroxidase-labeled anti-mouse IgG (Jackson) in an ELISA assay. Approximately 200 mAbs were selected that showed strong reactivity against the synthetic peptide. Hybridomas of interest were subcloned using limiting dilution.

To determine which antibodies could recognize the native, surface expressed molecule, the MAb's were screened against Sf9 insect cells infected with AcMNPV virus carrying human Eos L2 genomic DNA. These insect cells expressed Eos L2 (CKR-3) receptor on the cell surface, as judged by strong anti-FLAG staining of approximately 10% of cells. Staining was performed using M2 anti-FLAG antibody, followed by anti-mouse Ig-FITC (Jackson ImmunoResearch Laboratories, Inc.), and analyzed by fluorescence activated cell sorting, using FACScan analysis to quantitate expression. (Current Protocols in Immunology, Vol. 1, Coligan, J. et al., Eds., (John Wiley & Sons, Inc.; New York, NY).

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Approximately 33% of the anti-peptide hybridomas reacted with the Eos L2 transfected insect cells, with a staining pattern identical to that of the FLAG antibody, as determined by FACS analysis using anti-mouse Ig-FITC (Jackson ImmunoResearch Laboratories, Inc.) as second antibody. Untransfected insect cells stained with anti-FLAG were completely negative. Anti-peptide antibody also tested against untransfected cells, which were negative for staining.

10 MAb's that were found to stain the transfected insect cells were examined using FACS analysis for their reactivity with human eosinophils, peripheral blood lymphocytes, monocytes, neutrophils, and activated T cells (activated T cells; lymphocytes were treated with an anti-  
15 CD3 antibody to activate T cells). Cells were stained with mAb LS26-5H12 and then FITC-anti-mouse Ig (Jackson ImmunoResearch Laboratories, Inc.). Fc receptor binding was controlled for by using an excess of normal human serum.

20 All eosinophils were stained with a selected anti-Eos L2 mAb, LS26-5H12 (Figure 12A). Monocytes were weakly positive for immunofluorescence. A small proportion of lymphocytes were positive for staining (Figure 12B), and substantially all of the activated T cells were weakly  
25 stained with the antibody LS26-5H12 (not shown), suggesting that T cells express receptor which is upregulated upon T cell activation. Neutrophils were not significantly stained by LS26-5H12 antibody under the conditions of the assay. Based on the expected distribution of the Eos L2  
30 receptor, and that it functions in RANTES binding, MAb LS26-5H12 appears to recognize the naturally expressed form of this receptor. In addition to the LS26-5H12 MAb, - five additional Mabs behaved similarly.

The LS26-5H12 hybridoma was further purified by  
35 limiting dilution. In another experiment, highly purified

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leukocyte subsets (purified as described in Example 5) were stained with MAb LS26-5H12 and analyzed by flow cytometry (Figures 13A-13D). Staining profiles were representative of at least 4 experiments. T Cells were identified based on CD3 staining. Monocytes and neutrophils were identified by forward and side scatter.

Highly purified eosinophils stained strongly with LS26-5H12 (Figure 13A), suggesting abundant expression of the receptor on the surface of eosinophils, and consistent with a high receptor number determined by ligand binding and Scatchard analysis. Neutrophils, blood T cells, and monocytes showed little or no staining with this MAb (Figures 13B-13D). These latter results, using antibody from the recloned hybridoma, suggest CKR-3 is selectively expressed on eosinophils, and is not appreciably expressed on other leukocyte types tested. However, it is possible that a subset of T cells expresses the receptor.

#### Antibody Inhibition of Chemotaxis

Polyclonal antiserum (collected from the same mouse from which the LS26-5H12 MAb was made) were used in a chemotaxis assay, using butyric acid-differentiated HL-60 cells as described above, and culture inserts. (The insert forms an upper chamber when placed into a well of the microtiter dish). Chemotaxis of the butyric acid-differentiated HL-60 cells in response to 100 ng/ml of RANTES (in the lower chamber) was monitored. Chemotaxis in response to RANTES occurred to the same extent in a no serum control as in the presence of 1  $\mu$ l of normal mouse serum (placed in the upper chamber with cells). In contrast, in the presence of 1.0  $\mu$ l of antiserum (placed in the upper chamber with cells), RANTES-induced chemotaxis was inhibited by 40%. In a different test, using a polyclonal anti-peptide rabbit serum did not similarly inhibit chemotaxis.

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EXAMPLE 6Selection of Stable L1.2 Cell Transfectants

2% - 5% of transiently transfected COS, HEK-293 and CHO cells were surface positive as assessed using antibodies to FLAG-tagged receptor (see above), while substantial intracellular protein could be detected, suggesting inefficient protein trafficking. The L1.2 mouse pre-B cell line was used to select lines with higher levels of surface expression (see Figure 6) for further assessment of ligand binding specificity and signal transduction by CKR-3. This cell line has been used successfully for the study of other chemoattractant receptors (Honda, S., et al., *J. Immunol.*, 152: 4026-4035 (1994)), and the expression of transfected human chemokine receptors confers specific chemotactic ability to various ligands (see below).

To monitor surface expression of CKR-3, a monoclonal antibody (MAb) was produced to the N-terminal region of the receptor, by immunizing mice with a synthetic peptide having a sequence corresponding to the N-terminal 35 amino acids of CKR-3. Anti-peptide MAbs were detected by ELISA, and MAbs that recognize the native receptor were identified by their reactivity with human eosinophils, as well as their staining of transient transfectants.

25 Construction of CKR-3/pcDNA3

PCR was used to modify the CKR-3 gene contained in the 1.8 kb genomic fragment by inserting a HindIII restriction site and optimal Kozak sequence immediately 5' to the initiation codon. The coding region and 448 bp of 3'-untranslated region were inserted into the HindIII site of pcDNA3 (Invitrogen), placing the gene under the control of the human CMV immediate early gene promoter of the vector. The details of the construction of this FLAG-

tagged Eos L2 (CKR-3) receptor construct (also referred to herein as CKR-3/pcDNA3) are provided in Example 3.

#### Transfection and Stable Cell Line Selection

The murine pre-B lymphoma cell line L1.2 was obtained  
5 from Dr. Eugene Butcher (Stanford University) and  
maintained in RPMI-1640 supplemented with 10% bovine serum.  
20 µg of linearized, CKR-3/pcDNA3 was used to transfect the  
cell line as follows. L1.2 cells were washed twice in HBSS  
and resuspended in 0.8 ml of the same. The plasmid DNA was  
10 mixed with the cells and incubated for 10 minutes at room  
temperature then transferred to a 0.4 cm electroporation  
cuvette and a single pulse applied at 250 V, 960 µF. The  
electroporation was followed by a 10 minute incubation at  
room temperature. G418 was added to a final concentration  
15 of 0.8 mg/ml 48 hr post-transfection and the cells plated  
in 96 well plates at 25,000 cells/well. After 2-3 weeks  
under drug selection, G418 resistant cells were stained  
with 5H12 anti-receptor mAb (see below) and analyzed by  
FACScan®.

20 Lines with detectable surface staining were expanded  
and cloned several times by limiting dilution. Clones with  
the brightest surface staining were further analyzed by  
Northern hybridization to confirm the presence of  
transfected receptor as well as by RT-PCR using a T7 primer  
25 complementary to the pcDNA3 vector as the 5' primer and a  
CKR-3 specific primer as the 3' primer (not shown). No  
amplification was seen without addition of reverse  
transcriptase.

For transient transfection, 20 µg of supercoiled DNA  
30 was used in the electroporation exactly as described for  
stable cell line production. Cell surface staining was  
assessed after 48-72 hrs.

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L1.2 cells transfected with CKR-3/pcDNA3 were diluted to  $1 \times 10^6$  cells/ml in tissue culture media. n-butyric acid (sodium salt, Sigma Chemical Corp., Cat. No. B5887) was added to a final concentration of 5 mM (diluted from a 5 1M stock solution made in tissue culture media). Cells were grown overnight (18-24 hours) at 37°C, 5% CO<sub>2</sub> prior to use. Lower concentrations have been used successfully (e.g., 2.5 mM and 1 mM n-butyric acid). n-butyrate treatment has been reported to induce protein levels up to 10 about 10-fold relative to uninduced controls (see, e.g., 10 Palermo, D.P., et al., *J. Biotech.*, 19: 35-48 (1991) and references cited therein). CKR-3 mRNA levels driven by the human CMV immediate early gene promoter were elevated dramatically by n-butyrate treatment.

15 Monoclonal Antibody Production and Flow Cytometry

MAbs reactive with synthetic peptide were produced as described above in Example 5. MAbs were screened by ELISA as follows. 50 µl of peptide, at a concentration of 2 µg/ml in carbonate buffer, was used to coat NUNC 96-well 20 Maxisorp plates for at least 4 hours at 4°C. 300 µl/well of blocking buffer (PBS + 1% BSA) was added for at least 2 hours. Plates were washed four times with PBS/Tween 20, and 50 µl of MAb supernatant was added to each well and incubated at 37°C for one hour. Plates were washed four 25 times with PBS/Tween 20 and alkaline phosphatase-conjugated second antibody (Jackson ImmunoResearch Laboratories, West Grove PA) diluted 1:500 in PBS was added to each well. After an incubation at 37°C for 30 minutes, plates were washed four times with PBS/Tween 20. The substrate used 30 for the color reaction was p-nitrophenylphosphate dissolved in diethanolamine buffer (Bio-Rad). Plates were read at 410 nm on an ELISA reader.



To determine which anti-peptide MAb could recognize native, surface expressed CKR-3, the anti-peptide MAb were screened against transiently transfected cells and eosinophils. For MAb staining, cells were washed once with  
5 PBS, and resuspended in 100  $\mu$ l PBS containing 2% FCS, 0.1% sodium azide (FACS buffer), 5  $\mu$ g/ml purified antibody, 5  $\mu$ g/ml MOPC-21 IgG, isotype matched control MAb (Sigma) or 100  $\mu$ l hybridoma culture supernatant. After 30 min at 4°C, cells were washed twice in FACS buffer, and resuspended in  
10 100  $\mu$ l of FITC-conjugated affinity purified F(ab')<sub>2</sub> goat anti-mouse IgG (Jackson). After incubating for 30 minutes at 4°C, cells were washed twice in FACS buffer and analyzed by FACScan® to determine the level of surface expression. Propidium iodide was used to exclude dead cells.

15 Surface Expression of Receptor on Stable Transfectants of the L1.2 Cell Line

Figure 14A shows detectable surface staining of the transiently transfected receptor on a subpopulation of L1.2 cells, using an anti-receptor MAb, LS26-5H12.  
20 Untransfected L1.2 cells were negative (Figure 14B). A stable cell line was constructed by limiting dilution cloning of the transfectants and selection for higher surface staining as described above. This process yielded lines that had much higher levels of receptor expression  
25 (Figure 14C). Northern blot analysis confirmed the presence of transfected CKR-3 mRNA in one of the subclones, designated E5, and its absence in untransfected L1.2 cells (not shown).

EXAMPLE 7Ligand Binding Specificity of Stable L1.2 TransfectantsChemokines

- Recombinant human chemokines were obtained from
- 5 Peprotech, Inc. (Rocky Hill, NJ), except for human eotaxin which was synthesized using solid-phase methods that were optimized and adapted to a fully automated peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) as described (Clark-Lewis, I., et al.,
- 10 *Biochemistry*, 30: 3128-3135 (1991)). Human eotaxin is also commercially available from Peprotech.

<sup>125</sup>I-Labeling

- <sup>125</sup>I-labeled eotaxin was produced using the Bolton Hunter reagent (NEN), as described (Coligan, J.E., et al.,
- 15 *Eds.*, 1992, *Current Protocols in Immunology* (New York: John Wiley and Sons)). The specific activity of radiolabeled eotaxin was calculated to be 180 Ci/mM.

Ligand Binding

- Chemokine binding to target cells was carried out
- 20 using a modification of a previously reported method (Van Riper, G., et al., *J. Exp. Med.* 177: 851-856 (1993)). Cells were washed once in PBS and resuspended in binding buffer (50 mM HEPES, pH 7.5, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.5% BSA and 0.05% azide) at a concentration of 1 x 10<sup>7</sup>/ml.
- 25 Aliquots of 50  $\mu$ l (5 x 10<sup>5</sup> cells) were dispensed into microfuge tubes, followed by the addition of cold competitor and radiolabeled chemokines. The final reaction volume was 200  $\mu$ l. Non-specific binding was determined by incubating cells with radiolabeled chemokines in the
- 30 presence of 250-500 nM of unlabeled chemokines. After 60 minutes incubation at room temperature, the cells were

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washed 3 times with 1 ml of binding buffer containing 0.5 M NaCl. Cell pellets were then counted.

Competition is presented as the percentage of specific binding as calculated by  $100(S-B)/(T-B)$ , where S is the radioactivity of the sample, B as background binding and T as total binding without competitors. Background binding was obtained by incubating cells with radiolabeled chemokine and at least 400-fold excess of unlabeled chemokines. The total binding of eotaxin to E5 cells was 11611  $\pm$  119 cpm and background binding 2248  $\pm$  745 cpm. The total binding of eotaxin to eosinophils was 7866  $\pm$  353 cpm and background binding 1148  $\pm$  518 cpm. Duplicates were used throughout the experiments and the standard deviations were always less than 10% of the mean. All experiments were repeated at least three times. Curve fit was calculated by KaleidaGraph software.

The E5 cell line described in Example 6 was tested for its ability to bind radiolabeled eotaxin. Cells were incubated with 0.6 nM  $^{125}\text{I}$ -labeled eotaxin and various concentrations of cold competitor. Figure 15A shows that the transfected cells bound  $^{125}\text{I}$ -labeled eotaxin specifically and with high affinity. Scatchard analysis of the binding data indicated a dissociation constant ( $K_d$ ) of 1.5 nM (Figure 15C), similar to the value of 0.5 nM obtained using purified human eosinophils (Figure 15D). In addition, both RANTES and MCP-3 were able to specifically compete for binding. None of the other chemokines tested, including MIP-1 $\alpha$ , MIP-1 $\beta$ , or IL-8 were able to specifically compete for radiolabeled ligand (Figure 16).

### 30 Chemotaxis Assays

Chemotaxis with human eosinophils was assessed using a modification of a transendothelial assay (Carr, M.W. et al., *Proc. Natl. Acad. Sci. USA*, 91: 3652-3656 (1994)).

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The endothelial cells used for this assay were the endothelial cell line ECV 304, obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.). Endothelial cells were cultured on 6.5-mm diameter Biocoat® Transwell tissue culture inserts (Costar Corp., Cambridge MA) with a 3.0  $\mu$ M pore size. Culture media for ECV 304 cells consisted of M199 + 10% Fetal Calf Serum, L-glutamine, and antibiotics.

- Assay media consisted of equal parts RPMI 1640 and M199, with 0.5% BSA. 24 hours before the assay,  $2 \times 10^5$  ECV 304 cells were plated onto each insert of the 24-well chemotaxis plate, and incubated at 37 °C. Chemotactic factors (diluted in assay medium) were added to the 24-well tissue culture plates in a final volume of 600  $\mu$ l.
- Endothelial-coated tissue culture inserts were inserted into each well and  $10^6$  cells were added to the top chamber in a final volume of 100  $\mu$ l. The plate was incubated at 37°C in 5% CO<sub>2</sub>/95% air for 4 hours.

- The cells that had migrated to the bottom chamber were counted using flow cytometry. 500  $\mu$ l of the cell suspension from the lower chamber was placed in a tube, and relative cell counts were obtained by acquiring events for a set time period of 30 seconds. This counting method was found to be reproducible, and enables gating on the leukocytes and the exclusion of debris or other cells. Counts obtained in this way match closely those obtained by counting with a microscope.

- The same assay was used to assess chemotaxis of L1.2 cells or L1.2 receptor transfectant cell lines, except that endothelial cells were not used to coat the Biocoat® Transwell tissue culture inserts.

CKR-3 expression in L1.2 cells confers chemotactic responsiveness for eotaxin, RANTES and MCP-3

L1.2 receptor transfectants were tested for their ability to migrate in response to a panel of chemokines over a range of concentrations. The CKR-3 expressing cell line E5 showed a chemotactic response to eotaxin, RANTES, and MCP-3 with a peak response to eotaxin at 100 ng/ml, although specific migration could be detected as low as 10 ng/ml (Figure 17A). While a response to RANTES was evident at both 10 ng/ml and 100 ng/ml, the magnitude of the response was not as great as with eotaxin. MCP-3 appeared to be a less potent chemoattractant on the E5 cell line than on eosinophils, with no detectable migration below 100 ng/ml. No significant response to other chemokines tested was seen with this cell line. In other control experiments, cells did not migrate to the bottom chamber when chemokine was added to the top well alone, confirming that cell migration was chemotactic rather than chemokinetic (not shown).

The untransfected L1.2 cell line did not migrate in response to any chemokines tested (Figure 17B). Indeed, a striking feature of the L1.2 cell line was the very low background chemotaxis to non-specific ligands. As a specificity control, L1.2 cells transfected with IL-8 RB migrated specifically in response to IL-8 and GRO $\alpha$  (Figure 17C), as well as NAP-2 and ENA-78 (not shown), but not to other CXC or CC chemokines. Other chemokine receptors which were introduced into L1.2 cells by transfection also confer chemotactic ability to their specific ligands, including CKR-2 transfectants (which respond to MCP-1 and MCP-3), CKR-1 transfectants (which respond to MIP-1 $\alpha$ ), and IL-8 RA transfectants (which respond to IL-8) (not shown). Pertussis toxin completely abrogated the chemotactic response of both eosinophils and the CKR-3 transfectants to eotaxin indicated that the receptor was signaling through

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the G $\alpha$  subclass (Simon, M.I., et al., *Science*, 252: 802-808 (1991)) in both normal and transfected cells (not shown).

The chemotactic profile of eosinophils resembles that of  
CKR-3 transfectants

5 In order to assess whether the function of normal eosinophils resembled that of CKR-3 L1.2 transfectants, chemotaxis experiments were performed using eosinophils from a number of normal individuals (humans), having high levels of eosinophils (-6 to 8% of WBC) (purified as  
10 described in Example 5). Figures 18A-18B show two characteristic patterns of eosinophil chemotaxis observed in two different individuals. One pattern was characterized by a robust migration to eotaxin, and a lesser response to RANTES and MCP-3 (Figure 18A). The  
15 other pattern showed essentially equivalent chemotaxis in response to eotaxin, RANTES and MCP-3 (Figure 18B). These patterns were not due to variations in the assay, since within each individual, they were highly reproducible over a long period of time. MIP-1 $\alpha$  showed only weak chemotactic  
20 activity for eosinophils in the second class of individuals.

EXAMPLE 8

Cloning of a cDNA encoding Eos L2

Construction of an Eosinophil cDNA Library

25 Eosinophils were obtained from a patient (M.V.) diagnosed with idiopathic hyper-eosinophilic syndrome (Costa, J.J. et al., *J. Clin. Invest.*, 91: 2673 (1993)). RNA was isolated using a standard guanidinium isothiocyanate/cesium chloride method (In: *Current  
30 Protocols In Molecular Biology*, Vol. 1, Ausubel, F.M. et al., Eds., (John Wiley & Sons: New York, NY) page 4.2.2-4.2.3 (1991)). mRNA was obtained using Dynabeads® (Dynal,

Inc.), and the bacteriophage library was constructed using the SUPERSCRIPT™ Lambda System for cDNA Synthesis and  $\lambda$  Cloning (Gibco BRL, Life Technologies) which comes with  $\lambda$ gt22A, NotI-SalI arms.

#### 5 Library Screening

We screened approximately 750,000 bacteriophage plaques of the resulting human eosinophil cDNA library in duplicate. The probe used was a full-length radiolabeled cDNA probe (p4 cDNA) which encodes the MIP-1 $\alpha$ /RANTES receptor (CKR-1) (Gao et al., *J. Exp. Med.*, 177: 1421 (1993)). The p4 cDNA was cloned into the BamHI (5') and XhoI (3') sites of pCDNAI (Invitrogen). A BamHI-XhoI fragment of this clone (i.e., p4 cDNA in pCDNAI) was obtained by restriction digestion, and isolated using Gene Clean (Bio101). The fragment was labeled with  $^{32}$ P using a random primer labeling kit (Boehringer Mannheim Biochemicals).

Filters were prehybridized by incubation for two hours at 42 °C, in a solution of 50% formamide, 5 X SSC, 1X Denhardt's, 10% Dextran Sulfate, 20 mM TRIS, pH 7.5, 0.1% SDS (sodium dodecyl sulfate). Hybridization was performed overnight at 42 °C in the same solution. Eosinophil cDNA library filters were then washed two times with 2X SSC/0.1% SDS at room temperature, and two times with 2X SSC/0.1% SDS at 42 °C. Each wash was for 30 minutes. Filters were exposed overnight and positive plaques were picked in duplicate. Clones were further evaluated when positive in duplicate after the low stringency washes.

#### Characterization of cDNA Clones

30 Plaques were plaque purified, and DNA was isolated by a small scale phage lysis protocol (In: *Current Protocols In Molecular Biology*, Vol. 1, Suppl. 10, Ausubel, F.M. et

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al., Eds., (John Wiley & Sons: New York, NY), page 1.13.7 (1991). The bacteriophage DNA was digested with EcoRI (site in arm of vector) and NotI. The inserts released by digestion were visualized on a gel, and were found to be approximately 1.6 kb in length. The ~1.6 kb insert present in a plaque designated Mip-16 or M-16, was isolated using Gene Clean (Bio101), and was cloned into the EcoRI and NotI sites of Bluescript® vector KS (Stratagene), which had been digested with both EcoRI and NotI to produce asymmetric ends. The ligated plasmid was introduced into XL1-Blue *E. coli* cells (Stratagene) made competent as described by Hanahan (Hanahan, D., (1985), In: DNA Cloning, Volume 1, D.M. Glover, Ed. (IRL Press: Washington, D.C.), pp. 109-135).

Dideoxy sequencing of the M-16/Bluescript construct was performed using a dideoxynucleotide sequencing kit obtained from USB (United States Biochemical, Cleveland, OH). The nucleotide sequence of this clone was determined to encode a novel protein with a high degree of homology to the MIP-1α/RANTES receptor; however, from the sequence data, the clone did not appear to be full-length.

In order to identify a full-length clone, 15-20 additional plaques were isolated and purified, and the inserts present in the phage were characterized by restriction enzyme analysis and/or sequencing. Another λ clone, designated M31, which was isolated was found to contain a ~1.8 kb insert. The insert was cloned into the EcoRI and NotI sites of Bluescript® vector KS (Stratagene), and introduced into XL1-Blue *E. coli* cells (Stratagene) as described above. DNA sequencing of this clone (M31 insert in Bluescript, referred to as M31/Bluescript construct) was performed as described above, and revealed that it encoded a full-length receptor.

The M31 insert was released from the M31/Bluescript construct by digestion with EcoRI and NotI. The resulting



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fragment was isolated using Gene Clean (Bio101), and was inserted into the EcoRI and NotI sites of vector Ap'M9, which had been digested with both EcoRI and NotI to produce asymmetric ends. Vector Ap'M9 (de Fougerolles, A.R. et al., *J. Exp. Med.*, 177: 1187-1192 (1993)) is a derivative of CDM8 (Invitrogen) containing the  $\beta$ -lactamase from pBluescript and a polylinker from pSP64. The resulting construct, designated A31, was introduced into competent XL1-Blue cells.

10 The nucleotide sequence of the full-length cDNA and the predicted amino acid sequence of the encoded protein are shown in Figures 2A-2B (see also SEQ ID NO:3 and SEQ ID NO:4). The cDNA sequence shown in Figures 2A-2B was determined from clones A31 (bases 15-365 (numbering as in  
15 Figures 2A-2B)), and the M-16/Bluescript construct (bases 366 to 1152 (numbering as in Figures 2A-2B)). A comparison of the amino acid sequence of the novel receptor with other proteins revealed that the novel receptor and the MIP-1 $\alpha$ /RANTES receptor share 62% sequence identity, and the  
20 novel receptor and the MCP-1 receptor share 50.57% sequence identity. Sequence identity was determined using the Wisconsin UW GCG package (program gap), with the Needleman and Wunsch algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)).

#### 25 Northern Analysis

RNA for Northern analysis was obtained from a patient having hyper-eosinophilia. The eosinophils were isolated as described (Costa, J.J., et al., *J. Clin. Invest.*, 91: 2673 (1993)). Total eosinophil RNA was isolated using  
30 standard procedures (In: *Current Protocols In Molecular Biology*, Vol. 1, Ausubel, F. M. et al., Eds., (John Wiley & Sons: New York, NY) page 4.2.2-4.2.3 (1991)). The total RNA was fractionated on a 1% agarose gel, and then blotted

onto GeneScreen filters (New England Nuclear). Filters were probed at high stringency according to the manufacturer's protocol for high stringency washing of Gene Screen blots (New England Nuclear).

5        Several Northern blots were prepared. One involved probing with the EcoRI-NotI fragment of the M16/Bluescript construct, and others were probed with the EcoRI-NotI fragment from clone A31. Both EcoRI-NotI fragments include the 3' untranslated regions. Probes were labeled with <sup>32</sup>P  
10        using a random primer labeling kit (Boehringer Mannheim Biochemicals).

      The Northern blots each revealed a very strong signal of approximately 1.8 kb in total human eosinophil RNA. This result indicates that the A31 RNA is expressed at very  
15        high levels in eosinophils from this patient.

#### EXAMPLE 9

#### Expression of cDNA encoding Eos L2 receptor and Ligand Binding Studies

##### Constructs

20        Vectors A31 (described above) and A31-pcDNA3 were used for expression and binding analyses. To construct A31-pcDNA3, vector A31 was digested with EcoRI and NotI, the -1.8 kb insert was isolated using Gene Clean (Bio101), and was inserted into the EcoRI and NotI sites of vector  
25        pcDNA-3 (Invitrogen), which had been digested with both EcoRI and NotI. The ligated construct, designated A31-pcDNA3, was introduced into competent XL1-Blue cells.

##### Transient Transfections

30        Transient transfections using A31 in the kidney cell line 293 initially suggested high affinity binding of A31 with radioactive RANTES. These initial binding studies

have been difficult to reproduce. Accordingly, stable cell lines have subsequently been produced with A31/pcDNA3 stably integrated into both RBL (rat basophilic leukemia) and 293 cells. RBL cells (Accession No. ATCC CRL 1378) were obtained from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, and 293 cells (Accession No. ATCC CRL 1573) were a gift from I. Charo, Gladstone Cardiovascular Institute.

#### Stable Cell Lines

Stable cell lines were constructed as follows. A31-pcDNA3 was linearized by digestion with NotI. The linearized plasmid was introduced into RBL and 293 cells by electroporation. Confluent 293 and RBL cells growing in 100 X 20 mm plates were trypsinized, resuspended in 1 cc of phosphate buffered saline (PBS) and electroporated in a 0.4 cm cuvette (BioRad) with settings of 960 microfarads and 250 volts. Stable transfectants were isolated by positive selection in medium containing geneticin. Specifically, the cells were first cultured in DMEM (BRL), 10% fetal calf serum for several days, and then were switched to DMEM, 10% fetal calf serum with 0.9 mg/cc of Geneticin (BRL). (DMEM, Dulbecco's Modified Eagle's Medium). After 3 weeks, surviving colonies were isolated sterilely with cloning cylinders, and individual clones were grown in individual wells in DMEM, 10% fetal calf serum with 0.9 mg/cc of Geneticin (BRL).

Surviving clones which expressed A31 RNA at high levels were detected by Northern analysis. 120 stable transfectants of the RBL line, and 38 stable transfectants of the 293 cell line, were screened. Specifically, RNA from individual clones was isolated using the acid phenol method (Chomczynski, P. and N. Sacchi, *Anal. Biochem.*, 162: 156-159 (1987)). RNA was fractionated by electrophoresis, blotted onto GeneScreen (New England Nuclear), and Northern

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blots were probed according to the manufacturer's suggestion for high stringency wash. The EcoRI-NotI insert from plasmid A31 was isolated, radiolabeled with <sup>32</sup>P using the random primer labeling kit (Boehringer Mannheim Biochemicals), and used as a probe. RNA was quantified by ethidium bromide staining on gels. Untransfected 293 or RBL cells were used as negative controls for the corresponding transfectants.

Stable cell lines designated A31-293-#8, A31-293-#9, A31-293-#17, and A31-293-#20 were subsequently found to express A31 RNA at very high levels relative to other lines. Clone A31-293-#20 which highly expresses the A31 message by Northern analysis, was selected for further study.

One RBL line was found to express low-medium amounts of RNA, but did not appear to bind RANTES under the conditions used (not shown).

#### Ligand Binding

Stable clone A31-293-#20 was grown in quantities sufficient for binding assays. In particular, cells were grown in 100 mm plates in DMEM, 10% fetal calf serum, 0.9 mg/cc geneticin. Plates were grown to confluence, and membranes were prepared as follows. Culture medium was removed, and the cells were washed with phosphate buffered saline. Cells were harvested by washing with TEN (40 mM TRIS, pH 7.5, 1 mM EDTA, and 150 mM NaCl). The cells were frozen in liquid nitrogen, thawed at room temperature, and the membrane fraction was collected by centrifugation in a conical tube for 10 minutes at 18,000 rpm. Each binding point was determined using one-half of the membranes harvested from a single 100 mm plate grown to confluence.

<sup>125</sup>I-labeled RANTES was purchased from New England Nuclear, and cold RANTES was purchased from Peprotech

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(Princeton, N.J.). <sup>125</sup>I-labeled MCP-3 was a gift from New England Nuclear, and cold MCP-3 was a gift from J. Van Damme, Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium (see also, Opdenakker, G. et al., *Biochem. Biophys. Res. Commun.*, 191(2): 535-542 (1993)). Binding assays were performed as described by Van Riper, G. et al., *J. Exp. Med.*, 177: 851 (1993), with the following modifications. In particular, the binding to membranes of 0.125 nanomolar of <sup>125</sup>I-RANTES was performed in the presence of varying concentrations of unlabeled ligand. Binding buffer was 50 mM Hepes, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.5% BSA, pH 7.2. Radiolabeled and cold ligand were added simultaneously to the membranes (see above), and incubated for 1.5 hours at room temperature. The binding reaction was added to 2 cc of wash buffer (0.5 M NaCl, 50 mM Hepes, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.5% BSA, pH 7.2), mixed by vortexing, and then placed on polyethyleneimine-treated Whatman GFC filters. Filters were washed with an additional two ccs of wash buffer. Activity retained on filters after washing was determined by scintillation counting. Filters were placed in 5 cc of scintillation fluid and were then counted in a miniaxi-beta liquid scintillation counter (United Technologies, Packard, Downers Grove, IL). All points were determined in triplicate, except for the point at 2 nM, which was determined in duplicate.

The results of the assay indicated high affinity binding of RANTES to the receptor encoded by the A31 clone (Figure 19). Scatchard analysis of the data indicated a K<sub>d</sub> of ~ 2.5 nM for RANTES, which is what is expected in normal cells.

Binding of MCP-3 to membranes from clone A31-293-#20 was also assessed using the ligand binding assay described above for RANTES binding to A31-293-#20 membranes (Figure

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20). Binding reactions contained 0.125 nanomolar  $^{125}\text{I}$ -labeled MCP-3.

In addition, specificity of binding was assessed by determining the extent to which labeled MCP-3 (bound in the absence of cold MCP-3), could be displaced by cold MCP-3 (Figure 21). All points were taken in duplicate.

The MCP-3 bound to membranes from untransfected cells could not be displaced by  $^{125}\text{I}$ -labeled MCP-3, indicating non-specific binding. In comparison, the MCP-3 bound to membranes from A31-293-#20 cells could be displaced by hot MCP-3, which is indicative of specific binding.

The results of these assays indicate that the receptor encoded by the A31 cDNA specifically binds human MCP-3.

#### Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Gerard, Craig  
Gerard, Norma P.  
Mackay, Charles  
Ponath, Paul D.  
Post, Theodore W.  
Qin, Shixin
- (ii) TITLE OF INVENTION: G-PROTEIN COUPLED RECEPTOR GENE
- (iii) NUMBER OF SEQUENCES: 18
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.
  - (B) STREET: Two Militia Drive
  - (C) CITY: Lexington
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02173
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/375,199
  - (B) FILING DATE: 19-JAN-1995
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Brook, David E.
  - (B) REGISTRATION NUMBER: 22,592
  - (C) REFERENCE/DOCKET NUMBER: LKS94-05A PCT
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617-861-6240
  - (B) TELEFAX: 617-861-9540

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1689 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATCCTTTTC CTGGCACCTC TGATATCCTT TTGAAATTCA TGTAAAGAA TCCCTAGGCT	60
GCTATCACAT GTGGCATCTT TGTGAGTAC ATGAATAAAT CAACTGGTGT GTTTTACGAA	120
GGATGATTAT GCTTCATTGT GGGATTGTAT TTTTCTTCTT CTATCACAGG GAGAAGTGAA	180
ATGACAACCT CACTAGATAC AGTTGAGACC TTTGGTACCA CATCCTACTA TGATGACGTG	240
GGCCTGCTCT GTGAAAAAGC TGATACCAGA GCACTGATGG CCCAGTTTGT GCCCCCGCTG	300
TACTCCCTGG TGTTCACTGT GGGCCTCTTG GGCAATGTGG TGGTGCTGAT GATCCTCATA	360
AAATACAGGA GGCTCCGAAT TATGACCAAC ATCTACCTGC TCAACCTGGC CATTTCCGAC	420
CTGCTCTTCC TCGTCACCCCT TCCATTCTGG ATCCACTATG TCAGGGGGCA TAACTGGGTT	480
TTTGGCCATG GCATGTGTAA GCTCCTCTCA GGGTTTTATC ACACAGGCTT GTACAGCGAG	540
ATCTTTTTCA TAATCCTGCT GACAATCGAC AGGTACCTGG CCATTGTCCA TGCTGTGTTT	600
GCCCTTCGAG CCCGGACTGT CACTTTTGGT GTCATCACCA GCATCGTCAC CTGGGGCCTG	660
GCAGTGCTAG CAGCTCTTCC TGAATTTATC TTCTATGAGA CTGAAGAGTT GTTTGAAGAG	720
ACTCTTTGCA GTGCTCTTTA CCCAGAGGAT ACAGTATATA GCTGGAGGCA TTTCCACACT	780
CTGAGAATGA CCATCTTCTG TCTCGTTCTC CCTCTGCTCG TTATGGCCAT CTGCTACACA	840
GGAATCATCA AAACGCTGCT GAGGTGCCCC AGTAAAAAAA AGTACAAGGC CATCCGGCTC	900
ATTTTTGTCA TCATGGCGGT GTTTTTTCAAT TTCTGGACAC CCTACAATGT GGCTATCCTT	960
CTCTCTTCCT ATCAATCCAT CTTATTTGGA AATGACTGTG AGCGGACGAA GCATCTGGAC	1020
CTGGTCATGC TGGTGACAGA GGTGATCGCC TACTCCCACT GCTGCATGAA CCGGGTGATC	1080
TACGCCTTTG TTGGAGAGAG GTTCCGGAAG TACCTGCGCC ACTTCTTCCA CAGGCACTTG	1140
CTCATGCACC TGGGCAGATA CATCCCATTG CTTCTTAGTG AGAAGCTGGA AAGAACCAGC	1200
TCTGTCTCTC CATCCACAGC AGAGCCGGAA CTCTCTATTG TGTTTtaggt AGATGCAGAA	1260
AATTGCCTAA AGAGGAAGGA CCAAGGAGAT NAAGCAAACA CATTAGCCT TCCCACTCA	1320
CCTCTAAAC AGTCCTTCAA ACCTTCCAGT GCAACACTGA AGCTCTTAAG ACACTGAAAT	1380
ATACACACAG CAGTAGCAGT AGATGCATGT ACCCTAAGGT CATTACCACA GGCCAGGGCT	1440
GGGCAGCGTA CTCATCATCA ACCTAAAAAG CAGAGCTTTG CTTCTCTCTC TAAAATGAGT	1500
TACCTATATT TTAATGCACC TGAATGTTAG ATAGTTACTA TATGCCGCTA CAAAAGGTA	1560
AAACTTTTTA TATTTTATAC ATTAACCTCA GCCAGCTATT ATATAAATAA AACATTTTCA	1620
CACAATACAA TAAGTTAACT ATTTTATTTT CTAATGTGCC TAGTTCTTTC CCTGCTTAAT	1680
GAAGGCTT	1689



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## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 355 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Thr Thr Ser Leu Asp Thr Val Glu Thr Phe Gly Thr Thr Ser Tyr
 1      5      10      15
Tyr Asp Asp Val Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala Leu
 20      25      30
Met Ala Gln Phe Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Phe Gly
 35      40      45
Leu Leu Gly Asn Val Val Val Val Met Ile Leu Ile Lys Tyr Arg Arg
 50      55      60
Leu Arg Ile Met Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp
 65      70      75      80
Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg Gly
 85      90      95
His Asn Trp Val Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly Phe
100      105      110
Tyr His Thr Gly Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu Thr
115      120      125
Ile Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu Arg Ala
130      135      140
Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly Leu
145      150      155      160
Ala Val Leu Ala Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu Glu
165      170      175
Leu Phe Glu Glu Thr Leu Cys Ser Ala Leu Tyr Pro Glu Asp Thr Val
180      185      190
Tyr Ser Trp Arg His Phe His Thr Leu Arg Met Thr Ile Phe Cys Leu
195      200      205
Val Leu Pro Leu Leu Val Met Ala Ile Cys Tyr Thr Gly Ile Ile Lys
210      215      220
Thr Leu Leu Arg Cys Pro Ser Lys Lys Lys Tyr Lys Ala Ile Arg Leu
225      230      235      240
Ile Phe Val Ile Met Ala Val Phe Phe Ile Phe Trp Thr Pro Tyr Asn
245      250      255

```

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Val Ala Ile Leu Leu Ser Ser Tyr Gln Ser Ile Leu Phe Gly Asn Asp  
 260 265 270  
 Cys Glu Arg Thr Lys His Leu Asp Leu Val Met Leu Val Thr Glu Val  
 275 280 285  
 Ile Ala Tyr Ser His Cys Cys Met Asn Pro Val Ile Tyr Ala Phe Val  
 290 295 300  
 Gly Glu Arg Phe Arg Lys Tyr Leu Arg His Phe Phe His Arg His Leu  
 305 310 315 320  
 Leu Met His Leu Gly Arg Tyr Ile Pro Phe Leu Pro Ser Glu Lys Leu  
 325 330 335  
 Glu Arg Thr Ser Ser Val Ser Pro Ser Thr Ala Glu Pro Glu Leu Ser  
 340 345 350  
 Ile Val Phe  
 355

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1193 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 92..1156

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTGTGCTTAT CCGGGCAAGA ACTTATOGAA ATACAATAGA AGACCCACGC GTCCGGTTTT 60  
 TACTTAGAAG AGATTTTCAG GGAGAAGTGA A ATG ACA ACC TCA CTA GAT ACA 112  
 Met Thr Thr Ser Leu Asp Thr  
 1 5  
 GTT GAG ACC TTT GGT ACC ACA TCC TAC TAT GAT GAC GTG GGC CTG CTC 160  
 Val Glu Thr Phe Gly Thr Thr Ser Tyr Tyr Asp Asp Val Gly Leu Leu  
 10 15 20  
 TGT GAA AAA GCT GAT ACC AGA GCA CTG ATG GCC CAG TTT GTG CCC CCG 208  
 Cys Glu Lys Ala Asp Thr Arg Ala Leu Met Ala Gln Phe Val Pro Pro  
 25 30 35  
 CTG TAC TCC CTG GTG TTC ACT GTG GCC CTC TTG GCC AAT GTG GTG GTG 256  
 Leu Tyr Ser Leu Val Phe Thr Val Gly Leu Leu Gly Asn Val Val Val  
 40 45 50 55  
 GTG ATG ATC CTC ATA AAA TAC AGG AGG CTC CGA ATT ATG ACC AAC ATC 304  
 Val Met Ile Leu Ile Lys Tyr Arg Arg Leu Arg Ile Met Thr Asn Ile  
 60 65 70

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TAC	CTG	CTC	AAC	CTG	GCC	ATT	TCG	GAC	CTG	CTC	TTC	CTC	GTC	ACC	CTT	352
Tyr	Leu	Leu	Asn	Leu	Ala	Ile	Ser	Asp	Leu	Leu	Phe	Leu	Val	Thr	Leu	
			75					80					85			
CCA	TTC	TGG	ATC	CAC	TAT	GTC	AGG	GGG	CAT	AAC	TGG	GTT	TTT	GGC	CAT	400
Pro	Phe	Trp	Ile	His	Tyr	Val	Arg	Gly	His	Asn	Trp	Val	Phe	Gly	His	
		90					95					100				
GGC	ATG	TGT	AAG	CTC	CTC	TCA	GGG	TTT	TAT	CAC	ACA	GGC	TTG	TAC	AGC	448
Gly	Met	Cys	Lys	Leu	Leu	Ser	Gly	Phe	Tyr	His	Thr	Gly	Leu	Tyr	Ser	
	105					110					115					
GAG	ATC	TTT	TTC	ATA	ATC	CTG	CTG	ACA	ATC	GAC	AGG	TAC	CTG	GCC	ATT	496
Glu	Ile	Phe	Phe	Ile	Ile	Leu	Leu	Thr	Ile	Asp	Arg	Tyr	Leu	Ala	Ile	
	120				125					130					135	
GTC	CAT	GCT	GTG	TTT	GCC	CTT	CGA	GCC	CGG	ACT	GTC	ACT	TTT	GGT	GTC	544
Val	His	Ala	Val	Phe	Ala	Leu	Arg	Ala	Thr	Val	Thr	Phe	Gly	Val		
				140					145					150		
ATC	ACC	AGC	ATC	GTC	ACC	TGG	GGC	CTG	GCA	GTG	CTA	GCA	GCT	CTT	CCT	592
Ile	Thr	Ser	Ile	Val	Thr	Trp	Gly	Leu	Ala	Val	Leu	Ala	Ala	Leu	Pro	
			155					160					165			
GAA	TTT	ATC	TTC	TAT	GAG	ACT	GAA	GAG	TTG	TTT	GAA	GAG	ACT	CTT	TGC	640
Glu	Phe	Ile	Phe	Tyr	Glu	Thr	Glu	Glu	Leu	Phe	Glu	Glu	Thr	Leu	Cys	
		170					175					180				
AGT	GCT	CTT	TAC	CCA	GAG	GAT	ACA	GTA	TAT	AGC	TGG	AGG	CAT	TTC	CAC	688
Ser	Ala	Leu	Tyr	Pro	Glu	Asp	Thr	Val	Tyr	Ser	Trp	Arg	His	Phe	His	
	185					190					195					
ACT	CTG	AGA	ATG	ACC	ATC	TTC	TGT	CTC	GTT	CTC	CCT	CTG	CTC	GTT	ATG	736
Thr	Leu	Arg	Met	Thr	Ile	Phe	Cys	Leu	Val	Leu	Pro	Leu	Leu	Val	Met	
	200				205					210					215	
GCC	ATC	TGC	TAC	ACA	GGA	ATC	ATC	AAA	ACG	CTG	CTG	AGG	TGC	CCC	AGT	784
Ala	Ile	Cys	Tyr	Thr	Gly	Ile	Ile	Lys	Thr	Leu	Leu	Arg	Cys	Pro	Ser	
				220					225					230		
AAA	AAA	AAG	TAC	AAG	GCC	ATC	CGG	CTC	ATT	TTT	GTC	ATC	ATG	GCG	GTG	832
Lys	Lys	Lys	Tyr	Lys	Ala	Ile	Arg	Leu	Ile	Phe	Val	Ile	Met	Ala	Val	
			235					240					245			
TTT	TTC	ATT	TTC	TGG	ACA	CCC	TAC	AAT	GTG	GCT	ATC	CTT	CTC	TCT	TCC	880
Phe	Phe	Ile	Phe	Trp	Thr	Pro	Tyr	Asn	Val	Ala	Ile	Leu	Leu	Ser	Ser	
		250					255					260				
TAT	CAA	TCC	ATC	TTA	TTT	GGA	AAT	GAC	TGT	GAG	CGG	AGC	AAG	CAT	CTG	928
Tyr	Gln	Ser	Ile	Leu	Phe	Gly	Asn	Asp	Cys	Glu	Arg	Ser	Lys	His	Leu	
	265					270					275					
GAC	CTG	GTC	ATG	CTG	GTG	ACA	GAG	GTG	ATC	GCC	TAC	TCC	CAC	TGC	TGC	976
Asp	Leu	Val	Met	Leu	Val	Thr	Glu	Val	Ile	Ala	Tyr	Ser	His	Cys	Cys	
	280				285					290					295	
ATG	AAC	CCG	GTG	ATC	TAC	GCC	TTT	GTT	GGA	GAG	AGG	TTC	CGG	AAG	TAC	1024
Met	Asn	Pro	Val	Ile	Tyr	Ala	Phe	Val	Gly	Glu	Arg	Phe	Arg	Lys	Tyr	
				300					305					310		

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CTG CGC CAC TTC TTC CAC AGG CAC TTG CTC ATG CAC CTG GGC AGA TAC	1072
Leu Arg His Phe Phe His Arg His Leu Leu Met His Leu Gly Arg Tyr	
315 320 325	
ATC CCA TTC CTT CCT AGT GAG AAG CTG GAA AGA ACC AGC TCT GTC TCT	1120
Ile Pro Phe Leu Pro Ser Glu Lys Leu Glu Arg Thr Ser Ser Val Ser	
330 335 340	
CCA TCC ACA GCA GAG CCG GAA CTC TCT ATT GTG TTT TAGGTAGATC	1166
Pro Ser Thr Ala Glu Pro Glu Leu Ser Ile Val Phe	
345 350 355	
CAGAAAATTG CCTAAAGAGG AAGGACC	1193

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 355 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Thr Ser Leu Asp Thr Val Glu Thr Phe Gly Thr Thr Ser Tyr	
1 5 10 15	
Tyr Asp Asp Val Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala Leu	
20 25 30	
Met Ala Gln Phe Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Val Gly	
35 40 45	
Leu Leu Gly Asn Val Val Val Val Met Ile Leu Ile Lys Tyr Arg Arg	
50 55 60	
Leu Arg Ile Met Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp	
65 70 75 80	
Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg Gly	
85 90 95	
His Asn Trp Val Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly Phe	
100 105 110	
Tyr His Thr Gly Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu Thr	
115 120 125	
Ile Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu Arg Ala	
130 135 140	
Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly Leu	
145 150 155 160	
Ala Val Leu Ala Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu Glu	
165 170 175	
Leu Phe Glu Glu Thr Leu Cys Ser Ala Leu Tyr Pro Glu Asp Thr Val	
180 185 190	

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Tyr Ser Trp Arg His Phe His Thr Leu Arg Met Thr Ile Phe Cys Leu  
 195 200 205  
 Val Leu Pro Leu Leu Val Met Ala Ile Cys Tyr Thr Gly Ile Ile Lys  
 210 215 220  
 Thr Leu Leu Arg Cys Pro Ser Lys Lys Lys Tyr Lys Ala Ile Arg Leu  
 225 230 235 240  
 Ile Phe Val Ile Met Ala Val Phe Phe Ile Phe Trp Thr Pro Tyr Asn  
 245 250 255  
 Val Ala Ile Leu Leu Ser Ser Tyr Gln Ser Ile Leu Phe Gly Asn Asp  
 260 265 270  
 Cys Glu Arg Ser Lys His Leu Asp Leu Val Met Leu Val Thr Glu Val  
 275 280 285  
 Ile Ala Tyr Ser His Cys Cys Met Asn Pro Val Ile Tyr Ala Phe Val  
 290 295 300  
 Gly Glu Arg Phe Arg Lys Tyr Leu Arg His Phe Phe His Arg His Leu  
 305 310 315 320  
 Leu Met His Leu Gly Arg Tyr Ile Pro Phe Leu Pro Ser Glu Lys Leu  
 325 330 335  
 Glu Arg Thr Ser Ser Val Ser Pro Ser Thr Ala Glu Pro Glu Leu Ser  
 340 345 350  
 Ile Val Phe  
 355

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1116 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGGGAGAAG TGAAATGACA ACCTCACTAG ATACAGTTGA GACCTTTGGT ACCACATCCT	60
ACTATGATGA CGTGCGCCTG CTCTGTGAAA AAGCTGATAC CAGAGCACTG ATGGCCCACT	120
TTGTGCCCCC GCTGTACTCC CTGGTGTTC A CTGTGGGCCT CTTGGGCAAT GTGCTGGTGG	180
TGATGATCCT CATAAAATAC AGGAGGCTCC GAATTATGAC CAACATCTAC CTGCTCAACC	240
TGGCCATTTT GGACCTGCTC TTCCTCGTCA CCCTTCCATT CTGGATCCAC TATGTCAGGG	300
GGCATAACTG GGTTTTGGC CATGGCATGT GTAAGCTCCT CTCAGGGTTT TATCACACAG	360
GCTTGTAACG CGAGATCTTT TTCATAATCC TGCTGACAAT CGACAGGTAC CTGGCCATTG	420
TCCATGCTGT GTTTCGCCCTT CGAGCCCGGA CTGTCACTTT TGGTGTATC ACCAGCATCG	480
TCACCTGGGG CCTGGCAGTG CTAGCAGCTC TTCCTGAATT TATCTTCTAT GAGACTGAAG	540

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```

AGTTGTTTGA AGAGACTMTT TGCAGTGCTC TTTACCCAGA GGATACAGTA TATAGCTGGA      600
GSSATTTCCA CACTCTGAGA ATGACCATCT TCTGTCTCGT TCTCCCTCTG CTCGTTATGG      660
CCATCTGCTA CACAGGAATC ATCAAAACGC TGCTGAGGTG CCCCAGTAAA AAAAAGTACA      720
AGGCCATCCG GCTCATTTTT GTCATCATGG CGGTGTTTTT CATTTTCTGG ACACCCTACA      780
ATGTGGCTAT CTTTCTCTCT TSCYWYMAW YCATCTTATT TGGAAATGAC TGTGAGCCGM      840
NGARSMWYYK GGACCTGGTC ATGCTGGTGA CAGAGGTGAT CGCCTACTCC CACTGCTGCA      900
TGAACCCGGT GATCTACGCC TTTGTTGGAG AGAGGTTCCG GAAGTACCTG CGCCACTTST      960
TCCACAGGCA CTTGCTCATG CACCTGGGCA GATACATCCC ATTCCTTCCT AGTGAGAAGC     1020
TGGAAAGAAC CAGCTCTGTC TCTCCATCCA CAGCAGAGCC GGAACTCTCT ATTGTGTTTT     1080
AGGTAGATGC AGAAAATTGC CTAAAGAGGA AGGACC                                1116

```

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 355 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Thr Thr Ser Leu Asp Thr Val Glu Thr Phe Gly Thr Thr Ser Tyr
 1             5             10             15
Tyr Asp Asp Val Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala Leu
          20             25             30
Met Ala Gln Phe Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Val Gly
          35             40             45
Leu Leu Gly Asn Val Val Val Val Met Ile Leu Ile Lys Tyr Arg Arg
          50             55             60
Leu Arg Ile Met Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp
          65             70             75             80
Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg Gly
          85             90             95
His Asn Trp Val Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly Phe
          100            105            110
Tyr His Thr Gly Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu Thr
          115            120            125
Ile Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu Arg Ala
          130            135            140
Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly Leu
          145            150            155            160

```

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[illegible]

(2) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(1x) FEATURE:

- ```
(A) NAME/KEY: modified_base
(B) LOCATION: 19
(D) OTHER INFORMATION: /mod base= i
```

**(ix) FEATURE:**

- (A) NAME/KEY: modified\_base  
(B) LOCATION: 24  
(D) OTHER INFORMATION: /mod\_base= i

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:7:

**TACCTGCTSA ACCTGGCCNT GGCNG**

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## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /mod\_base= i

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 14
- (D) OTHER INFORMATION: /mod\_base= i

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCTGGCCNT GGCNGACCTM CTCTT

25

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 18
- (D) OTHER INFORMATION: /mod\_base= i

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACCGYTACC TGGCCATNGT CCAYGCC

27

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 10
- (D) OTHER INFORMATION: /mod\_base= i

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGCRTGGACN ATGCCAGGT ARCGGTC

27



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## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 1
  - (D) OTHER INFORMATION: /mod\_base= i
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 6
  - (D) OTHER INFORMATION: /mod\_base= i
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 16
  - (D) OTHER INFORMATION: /mod\_base= i
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 18
  - (D) OTHER INFORMATION: /mod\_base= i

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

NACCANRTTG TAGGGNRNCC ARMARAG

27

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 8
  - (D) OTHER INFORMATION: /mod\_base= i
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 10
  - (D) OTHER INFORMATION: /mod\_base= i
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 23
  - (D) OTHER INFORMATION: /mod\_base= i

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGTACCGNRN CCARMARAGR AGNARGAA

28

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## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 12
  - (D) OTHER INFORMATION: /mod\_base= i

- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 15
  - (D) OTHER INFORMATION: /mod\_base= i

- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 16
  - (D) OTHER INFORMATION: /mod\_base= i

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAAGCGGTAG ANSANNGGCT TGASGCA

27

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 4
  - (D) OTHER INFORMATION: /mod\_base= i

- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 7
  - (D) OTHER INFORMATION: /mod\_base= i

- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 8
  - (D) OTHER INFORMATION: /mod\_base= i

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGANSANNGG GTTGASGCAG CWGTC

25

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## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 48 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 16..48

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAGCTTCCAG CAGCC ATG GAC TAC AAG GAC GAC GAT GAC AAA GAA TTC  
Met Asp Tyr Lys Asp Asp Asp Asp Lys Glu Phe  
1 5 10

48

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 11 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Asp Tyr Lys Asp Asp Asp Asp Lys Glu Phe  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTAAGAATTC ACAACCTCAC TAGATAC

27

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CATAGTGCAT CCAGAATG

18

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CLAIMS

What is claimed is:

1. An isolated nucleic acid, wherein said isolated  
nucleic acid hybridizes to a nucleic acid selected  
5 from the group consisting of:
  - a) a nucleic acid having the sequence set forth in  
SEQ ID NO: 1 or a portion thereof comprising the  
coding sequence;
  - 10 b) a nucleic acid having the sequence set forth in  
SEQ ID NO: 3 or a portion thereof comprising the  
coding sequence ;
  - c) a nucleic acid having the sequence set forth in  
SEQ ID NO: 5 or a portion thereof comprising the  
coding sequence;
  - 15 d) the complement of any one of (a) through (c); and  
e) the RNA counterpart of any one of (a) through  
(d), wherein U is substituted for T.
2. The isolated nucleic acid of Claim 1, wherein the  
isolated nucleic acid is essentially pure.
- 20 3. The isolated nucleic acid of Claim 1, wherein the  
isolated nucleic acid hybridizes to any one of the  
nucleic acids set forth in a) through e) under  
stringent conditions.
- 25 4. The isolated nucleic acid of Claim 1, wherein the  
isolated nucleic acid encodes at least a functional  
portion of a mammalian chemokine receptor 3.
5. The isolated nucleic acid of Claim 4, wherein the  
mammal is a human.

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6. The isolated nucleic acid of Claim 4, wherein the isolated nucleic acid is recombinant.
7. An isolated, recombinant nucleic acid which encodes a mammalian chemokine receptor-3.
- 5 8. An isolated, recombinant nucleic acid of Claim 7, wherein the mammal is a primate.
9. An isolated, recombinant nucleic acid of Claim 8, wherein the primate is a human.
- 10 10. An isolated nucleic acid, wherein said nucleic acid encodes a polypeptide selected from the group consisting of:
  - a) a polypeptide having the amino acid sequence set forth in SEQ ID NO:2;
  - 15 b) a polypeptide having the amino acid sequence set forth in SEQ ID NO:4;
  - c) a polypeptide having the amino acid sequence set forth in SEQ ID NO:6; and
  - 20 d) a functional portion of any one of (a) through (c), said portion having at least one function characteristic of a mammalian C-C chemokine receptor.
11. The isolated nucleic acid of Claim 10, wherein said nucleic acid is essentially pure.
- 25 12. The isolated nucleic acid of Claim 10, which is a recombinant nucleic acid.
13. The nucleic acid of Claim 10, wherein the polypeptide binds one or more of RANTES, MCP-3, and eotaxin.

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14. The nucleic acid of Claim 10, wherein the polypeptide binds RANTES.
15. A recombinant construct comprising a recombinant nucleic acid which hybridizes to a nucleic acid selected from the group consisting of:
- a) a nucleic acid having the sequence set forth in SEQ ID NO: 1 or a portion thereof comprising the coding sequence;
  - b) a nucleic acid having the sequence set forth in SEQ ID NO: 3 or a portion thereof comprising the coding sequence ;
  - c) a nucleic acid having the sequence set forth in SEQ ID NO: 5 or a portion thereof comprising the coding sequence;
  - d) the complement of any one of (a) through (c); and
  - e) the RNA counterpart of any one of (a) through (d), wherein U is substituted for T.
16. The recombinant construct of Claim 15, wherein the recombinant nucleic acid is operably linked to an expression control sequence.
17. The recombinant construct of Claim 15, wherein the recombinant nucleic acid encodes at least a functional portion of a mammalian chemokine receptor-3.
18. The recombinant construct of Claim 17, wherein the mammal is a human.
19. A recombinant construct comprising a nucleic acid which encodes a mammalian chemokine receptor 3.
20. The recombinant construct of Claim 19, wherein the mammal is a primate.

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21. The recombinant construct of Claim 20, wherein the primate is a human.
22. A recombinant construct comprising a nucleic acid, wherein said nucleic acid encodes a polypeptide  
5 selected from the group consisting of:
- a) a polypeptide having the amino acid sequence set forth in SEQ ID NO:2;
  - b) a polypeptide having the amino acid sequence set forth in SEQ ID NO:4;
  - 10 c) a polypeptide having the amino acid sequence set forth in SEQ ID NO:6;
  - d) a functional portion of any one of (a) through (c), said portion having at least one function characteristic of a mammalian C-C chemokine  
15 receptor; and
  - e) a functional equivalent of any one of (a) through (c).
23. The recombinant construct of Claim 22, wherein the nucleic acid is operably linked to an expression  
20 control sequence.
24. An isolated, recombinant mammalian chemokine receptor 3 or portion thereof, said portion having at least one function characteristic of a mammalian chemokine receptor or an immunological property.
25. 25. An isolated, recombinant receptor of Claim 24, wherein the mammal is a human.
26. An essentially pure mammalian chemokine receptor 3 or portion thereof, having at least one function characteristic of a mammalian chemokine receptor, for  
30 example, exotoxin binding.

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27. A host cell containing an isolated, recombinant nucleic acid encoding a mammalian CKR-3 receptor protein or functional portion thereof.
- 5 28. A host cell of Claim 27, wherein the nucleic acid is operably linked to an expression control sequence, whereby the encoded receptor protein or functional portion thereof is expressed when the host cell is maintained under conditions suitable for expression.
- 10 29. A host cell of Claim 28, wherein the expressed protein binds one or more of RANTES, MCP-3, and eotaxin.
- 15 30. A fusion protein containing a mammalian chemokine receptor 3 protein or portion thereof, said portion having at least one functional characteristic of a mammalian chemokine receptor or an immunological property.
31. The fusion protein of Claim 30 wherein the mammalian chemokine receptor protein or portion thereof binds one or more of RANTES, MCP-3, and eotaxin.
- 20 32. The fusion protein of Claim 30, wherein the polypeptide binds RANTES.
33. The fusion protein of Claim 30, wherein the mammal is a human.
- 25 34. A nucleic acid construct, wherein said construct is an expression vector comprising a nucleic acid encoding a fusion protein of Claim 30, said nucleic acid comprising all or part of the coding sequence for a mammalian chemokine receptor 3 protein, wherein the



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coding sequence is under the control of an expression control sequence.

35. A method for producing a mammalian chemokine receptor 3 protein or functional portion thereof comprising  
5 maintaining a host cell containing a recombinant nucleic acid encoding said receptor or functional portion thereof under conditions suitable for expression of the nucleic acid, whereby the encoded protein is expressed and said receptor or portion  
10 thereof is produced.
36. A method for producing a mammalian chemokine receptor 3 protein or functional portion thereof comprising the following steps:
- 15 a) providing a recombinant nucleic acid construct comprising a nucleic acid which encodes all or part of a coding sequence for a mammalian chemokine receptor 3 protein wherein the coding sequence is operably linked to at least one expression control sequence;
- 20 b) introducing the construct into a suitable host cells; and
- c) maintaining the host cells in suitable medium under conditions whereby the nucleic acid is expressed.
- 25 37. The method of Claim 36, further comprising the step of isolating a mammalian chemokine receptor 3 protein or functional portion thereof from said cell or the medium of its growth.
- 30 38. An antibody or functional portion thereof which binds to a mammalian chemokine receptor 3 protein or portion of said receptor protein.

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39. An antibody of Claim 38, wherein the mammalian chemokine receptor 3 protein is a human mammalian chemokine receptor 3 protein.
- 5 40. A method of detecting a ligand of a mammalian chemokine receptor 3 protein or portion thereof comprising the steps of combining a compound to be tested with an active, isolated mammalian chemokine receptor 3 protein under conditions suitable for binding of ligand thereto, and detecting or measuring  
10 the formation of a complex between said compound and the active, isolated protein.
41. A method of identifying a ligand of a mammalian chemokine receptor 3 protein or portion thereof comprising the steps of:  
15 a) combining a compound to be tested with a host cell expressing an active, recombinant mammalian chemokine receptor 3 protein under conditions suitable for binding of ligand thereto; and  
b) detecting or measuring the formation of a complex  
20 between said compound and the active, isolated protein.
42. The method of Claim 41, wherein the formation of a complex is monitored by detecting or measuring a signalling activity or cellular response by said  
25 active receptor in response to binding of a ligand thereto.
43. A method of identifying an inhibitor of a mammalian chemokine receptor 3 protein or portion thereof comprising the steps of:  
30 a) combining a compound to be tested with a host cell expressing an active, recombinant mammalian

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chemokine receptor 3 protein under conditions suitable for binding of ligand thereto in the presence of a ligand; and

- 5           b) detecting or measuring the formation of a complex between said ligand and the active, isolated protein.
44. An inhibitor of at least one function characteristic of a mammalian chemokine receptor 3 protein.
- 10   45. An inhibitor of Claim 44, which is an antibody or portion thereof.
46. Antisense nucleic acid comprising a nucleic acid having a sequence that hybridizes to a nucleic acid having a sequence selected from the group consisting of:
- 15       a) SEQ ID NO: 1;  
       b) SEQ ID NO: 3;  
       c) SEQ ID NO: 5;  
       b) an RNA counterpart of any one of a) through c).
- 20   47. A method of modulating at least one function of a mammalian chemokine receptor 3 protein, comprising the step of contacting said protein with an inhibitor or promoter of at least one function of said protein.
- 25   48. A method for treating an inflammatory disease or condition, comprising administering to a mammal a therapeutically effective amount of an inhibitor of a mammalian chemokine receptor 3 protein, whereby inflammation is reduced.

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Sequence Range: 1 to 1689

```

      10      20      30      40
AAT CCT TTT CCT GGC ACC TCT GAT ATC CTT TTG AAA TTC ATG TTA AAG
50      60      70      80      90
AAT CCC TAG GCT GCT ATC ACA TGT GGC ATC TTT GTT GAG TAC ATG AAT
100     110     120     130     140
AAA TCA ACT GGT GTG TTT TAC GAA GGA TGA TTA TGC TTC ATT GTG GGA
150     160     170     180     190
TTG TAT TTT TCT TCT TCT ATC ACA GGG AGA AGT GAA ATG ACA ACC TCA
Met Thr Thr Ser>
200     210     220     230     240
CTA GAT ACA GTT GAG ACC TTT GGT ACC ACA TCC TAC TAT GAT CAC GTG
Leu Asp Thr Val Glu Thr Phe Gly Thr Thr Ser Tyr Tyr Asp Asp Val>
250     260     270     280
GGC CTG CTC TGT GAA AAA GCT GAT ACC AGA GCA CTG ATG GCC CAG TTT
Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala Leu Met Ala Gln Phe>
290     300     310     320     330
GTG CCC CCG CTC TAC TCC CTG GTG TTC ACT GTG GGC CTC TTG GGC AAT
Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Val Gly Leu Leu Gly Asn>
340     350     360     370     380
GTG GTG GTG GTG ATG ATC CTC ATA AAA TAC AGG AGG CTC CGA ATT ATG
Val Val Val Val Met Ile Leu Ile Lys Tyr Arg Arg Leu Arg Ile Met>
390     400     410     420     430
ACC AAC ATC TAC CTG CTC AAC CTG GGC ATT TCG GAC CTG CTC TTC CTC
Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Leu Phe Leu>
440     450     460     470     480
GTC ACC CTT CCA TTC TGC ATC CAC TAT GTC AGG GGG CAT AAC TGG GTT
Val Thr Leu Pro Phe Trp Jle His Tyr Val Arg Gly His Asn Trp Val>
490     500     510     520
TTT GGC CAT GGC ATG TGT AAG CTC CTC TCA GGG TTT TAT CAC ACA GGC
Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly Phe Tyr His Thr Gly>
530     540     550     560     570
TTG TAC AGC GAG ATC TTT TTC ATA ATC CTG CTG ACA ATC GAC AGG TAC
Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu Thr Ile Asp Arg Tyr>
580     590     600     610     620
CTG GCC ATT GTC CAT GCT GTG TTT GGC CTT CGA GCC CGG ACT GTC ACT

```

FIG. 1A

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Leu Ala Ile Val His Ala Val Phe Ala Leu Arg Ala Arg Thr Val Thr>  
 630 640 650 660 670  
 TTT GGT GTC ATC ACC ACC ATC GTC ACC TGG GGC CTG GCA GTG CTA GCA  
 Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly Leu Ala Val Leu Ala>  
 680 690 700 710 720  
 GCT CTT CCT GAA TTT ATC TTC TAT GAG ACT GAA GAG TTG TTT CAA GAG  
 Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu Glu Leu Phe Glu Glu>  
 730 740 750 760  
 ACT CTT TGC AGT GCT CTT TAC CCA GAG GAT ACA GTA TAT AGC TGG AGC  
 Thr Leu Cys Ser Ala Leu Tyr Pro Glu Asp Thr Val Tyr Ser Trp Arg>  
 770 780 790 800 810  
 CAT TTC CAC ACT CTC AGA ATG ACC ATC TTC TGT CTC GTT CTC CCT CTG  
 His Phe His Thr Leu Arg Met Thr Ile Phe Cys Leu Val Leu Pro Leu>  
 820 830 840 850 860  
 CTC GTT ATG GCC ATC TGC TAC ACA GGA ATC ATC AAA ACG CTC CTG AGC  
 Leu Val Met Ala Ile Cys Tyr Thr Gly Ile Ile Lys Thr Leu Leu Arg>  
 870 880 890 900 910  
 TGC CCC AGT AAA AAA AAG TAC AAG GCC ATC CGG CTC ATT TTT GTC ATC  
 Cys Pro Ser Lys Lys Lys Tyr Lys Ala Ile Arg Leu Ile Phe Val Ile>  
 920 930 940 950 960  
 ATG GCG GTG TTT TTC ATT TTC TGG ACA CCC TAC AAT GTG GCT ATC CTT  
 Met Ala Val Phe Phe Ile Phe Trp Thr Pro Tyr Asn Val Ala Ile Leu>  
 970 980 990 1000  
 CTC TCT TCC TAT CAA TCC ATC TTA TTT GGA AAT GAC TGT GAG CGG ACG  
 Leu Ser Ser Tyr Gln Ser Ile Leu Phe Gly Asn Asp Cys Glu Arg Thr>  
 1010 1020 1030 1040 1050  
 AAG CAT CTG GAC CTG GTC ATC CTG CTG ACA GAG GTG ATC GCC TAC TCC  
 Lys His Leu Asp Leu Val Met Leu Val Thr Glu Val Ile Ala Tyr Ser>  
 1060 1070 1080 1090 1100  
 CAC TGC TGC ATG AAC CCG GTG ATC TAC CCC TTT GTT GGA GAG ACG TTC  
 His Cys Cys Met Asn Pro Val Ile Tyr Ala Phe Val Gly Glu Arg Phe>  
 1110 1120 1130 1140 1150  
 CGG AAG TAC CTG CGC CAC TTC TTC CAC AGG CAC TTG CTC ATG CAC CTG  
 Arg Lys Tyr Leu Arg His Phe Phe His Arg His Leu Leu Met His Leu>  
 1160 1170 1180 1190 1200  
 GGC AGA TAC ATC CCA TTC CTT CCT AGT GAG AAG CTG GAA AGA ACC AGC  
 Gly Arg Tyr Ile Pro Phe Leu Pro Ser Glu Lys Leu Glu Arg Thr Ser>

FIG. 1B

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```

      1210      1220      1230      1240
      .      .      .      .
TCT GTC TCT CCA TCC ACA GCA GAG CCG GAA CTC TCT ATT GTG TTT TAG
Ser Val Ser Pro Ser Thr Ala Glu Pro Glu Leu Ser Ile Val Phe ***
1250      1260      1270      1280      1290
      .      .      .      .      .
GTA GAT GCA GAA AAT TGC CTA AAG AGG AAG GAC CAA CGA GAT NAA CCA
      1300      1310      1320      1330      1340
      .      .      .      .      .
AAC ACA TTA AGC CTT CCA CAC TCA CCT CTA AAA CAG TCC TTC AAA CCT
      1350      1360      1370      1380      1390
      .      .      .      .      .
TCC AGT GCA ACA CTG AAG CTC TTA AGA CAC TGA AAT ATA CAC ACA GCA
      1400      1410      1420      1430      1440
      .      .      .      .      .
GTA GCA GTA GAT GCA TGT ACC CTA AGG TCA TTA CCA CAG GCC AGG GCT
      1450      1460      1470      1480
      .      .      .      .
GGG CAG CGT ACT CAT CAT CAA CCT AAA AAG CAG AGC TTT GCT TCT CTC
1490      1500      1510      1520      1530
      .      .      .      .      .
TCT AAA ATG AGT TAC CTA TAT TTT AAT GCA CCT GAA TGT TAG ATA GTT
      1540      1550      1560      1570      1580
      .      .      .      .      .
ACT ATA TGC CCG TAC AAA AAG GTA AAA CTT TTT ATA TTT TAT ACA TTA
      1590      1600      1610      1620      1630
      .      .      .      .      .
ACT TCA GCC AGC TAT TAT ATA AAT AAA ACA TTT TCA CAC AAT ACA ATA
      1640      1650      1660      1670      1680
      .      .      .      .      .
AGT TAA CTA TTT TAT TTT CTA ATG TGC CTA GTT CTT TCC CTG CTT AAT
      .
CAA AAG CTT

```

FIG. 1C

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```

      10      20      30      40      50      60
      *      *      *      *      *      *
TTGTGCTTAT CCGGGCAAGA ACTTATCGAA ATACAATAGA AGACCCACGC GTCCGGTTTT

      70      80      90      100     110
      *      *      *      *      *
TACTTAGAAG AGATTTTCAG GGAGAAGTGA A ATG ACA ACC TCA CTA GAT ACA GTT
                      M  T  T  S  L  D  T  V>

      120     130     140     150     160
      *      *      *      *      *
GAG ACC TTT GGT ACC ACA TCC TAC TAT GAT GAC GTG GGC CTG CTC TGT
E  T  F  G  T  T  S  Y  Y  D  D  V  G  L  L  C>

      170     180     190     200     210
      *      *      *      *      *
GAA AAA GCT GAT ACC AGA GCA CTG ATG GCC CAG TTT GTG CCC CCG CTG
E  K  A  D  T  R  A  L  M  A  Q  F  V  P  P  L>

      220     230     240     250
      *      *      *      *
TAC TCC CTG GTG TTC ACT GTG GGC CTC TTG GGC AAT GTG GTG GTG GTG
Y  S  L  V  F  T  V  G  L  L  G  N  V  V  V  V>

260      270      280      290      300
      *      *      *      *      *
ATG ATC CTC ATA AAA TAC AGG AGG CTC CGA ATT ATG ACC AAC ATC TAC
M  I  L  I  K  Y  R  R  L  R  I  M  T  N  I  Y>

310      320      330      340      350
      *      *      *      *      *
CTG CTC AAC CTG GCC ATT TCG GAC CTG CTC TTC CTC GTC ACC CTT CCA
L  L  N  L  A  I  S  D  L  L  F  L  V  T  L  P>

      360     370     380     390     400
      *      *      *      *      *
TTC TGG ATC CAC TAT GTC AGG GGG CAT AAC TGG GTT TTT GGC CAT GGC
F  W  I  H  Y  V  R  G  H  N  W  V  F  G  H  G>

      410     420     430     440     450
      *      *      *      *      *
ATG TGT AAG CTC CTC TCA GGG TTT TAT CAC ACA GGC TTG TAC AGC GAG
M  C  K  L  L  S  G  F  Y  H  T  G  L  Y  S  E>

      460     470     480     490
      *      *      *      *
ATC TTT TTC ATA ATC CTG CTG ACA ATC GAC AGG TAC CTG GCC ATT GTC
I  F  F  I  I  L  L  T  I  D  R  Y  L  A  I  V>

500      510      520      530      540
      *      *      *      *      *
CAT GCT GTG TTT GCC CTT CGA GCC CGG ACT GTC ACT TTT GGT GTC ATC
H  A  V  F  A  L  R  A  R  T  V  T  F  G  V  I>

550      560      570      580      590
      *      *      *      *      *
ACC AGC ATC GTC ACC TGG GGC CTG GCA GTG CTA GCA GCT CTT CCT GAA
T  S  I  V  T  W  G  L  A  V  L  A  A  L  F  E>

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FIG. 2A

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      600      610      620      630      640
      *      *      *      *      *
TTT ATC TTC TAT GAG ACT GAA GAG TTG TTT GAA GAG ACT CTT TGC AGT
F   I   F   Y   E   T   E   E   L   F   E   E   T   L   C   S>

      650      660      670      680      690
      *      *      *      *      *
GCT CTT TAC CCA GAG GAT ACA GTA TAT AGC TGG AGG CAT TTC CAC ACT
A   L   Y   P   E   D   T   V   Y   S   W   R   H   F   H   T>

      700      710      720      730
      *      *      *      *
CTG AGA ATG ACC ATC TTC TGT CTC GTT CTC CCT CTG CTC GTT ATG GCC
L   R   M   T   I   F   C   L   V   L   P   L   L   V   M   A>

740      750      760      770      780
*      *      *      *      *
ATC TGC TAC ACA GGA ATC ATC AAA ACG CTG CTG AGG TGC CCC AGT AAA
I   C   Y   T   G   I   I   K   T   L   L   R   C   P   S   K>

790      800      810      820      830
*      *      *      *      *
AAA AAG TAC AAG GCC ATC CGG CTC ATT TTT GTC ATC ATG GCG GTG TTT
K   K   Y   K   A   I   R   L   I   F   V   I   M   A   V   F>

      840      850      860      870      880
      *      *      *      *      *
TTC ATT TTC TGG ACA CCC TAC AAT GTG GCT ATC CTT CTC TCT TCC TAT
F   I   F   W   T   P   Y   N   V   A   I   L   L   S   S   Y>

      890      900      910      920      930
      *      *      *      *      *
CAA TCC ATC TTA TTT GGA AAT GAC TGT GAG CGG AGC AAG CAT CTG GAC
Q   S   I   L   F   G   N   D   C   E   R   S   K   H   L   D>

      940      950      960      970
      *      *      *      *
CTG GTC ATG CTG GTG ACA GAG GTG ATC GCC TAC TCC CAC TGC TGC ATG
L   V   M   L   V   T   E   V   I   A   Y   S   H   C   C   M>

980      990      1000      1010      1020
*      *      *      *      *
AAC CCG GTG ATC TAC GCC TTT GTT GGA GAG AGG TTC CGG AAG TAC CTG
N   P   V   I   Y   A   F   V   G   E   R   F   R   K   Y   L>

1030      1040      1050      1060      1070
*      *      *      *      *
CGC CAC TTC TTC CAC AGG CAC TTG CTC ATG CAC CTG GGC AGA TAC ATC
R   H   F   F   H   R   H   L   L   M   H   L   G   R   Y   I>

      1080      1090      1100      1110      1120
      *      *      *      *      *
CCA TTC CTT CCT AGT GAG AAG CTG GAA AGA ACC AGC TCT GTC TCT CCA
P   F   L   P   S   E   K   L   E   R   T   S   S   V   S   P>

      1130      1140      1150      1160      1170
      *      *      *      *      *
TCC ACA GCA GAG CCG GAA CTC TCT ATT GTG TTT TAG G TAGATGCAGA
S   T   A   E   P   E   L   S   I   V   F   *>

      1180      1190
      *      *
AAATTGCCTA AAGAGGAAGG ACC

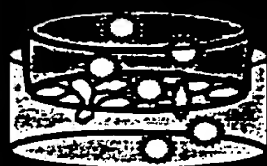
```

FIG. 2B



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# A simple leukocyte transendothelial assay for measuring chemotaxis



Insert (cells)

polycarbonate membrane, ECV304 endothelial cells

Bottom chamber (chemokine)

analysis

**FIG. 3**

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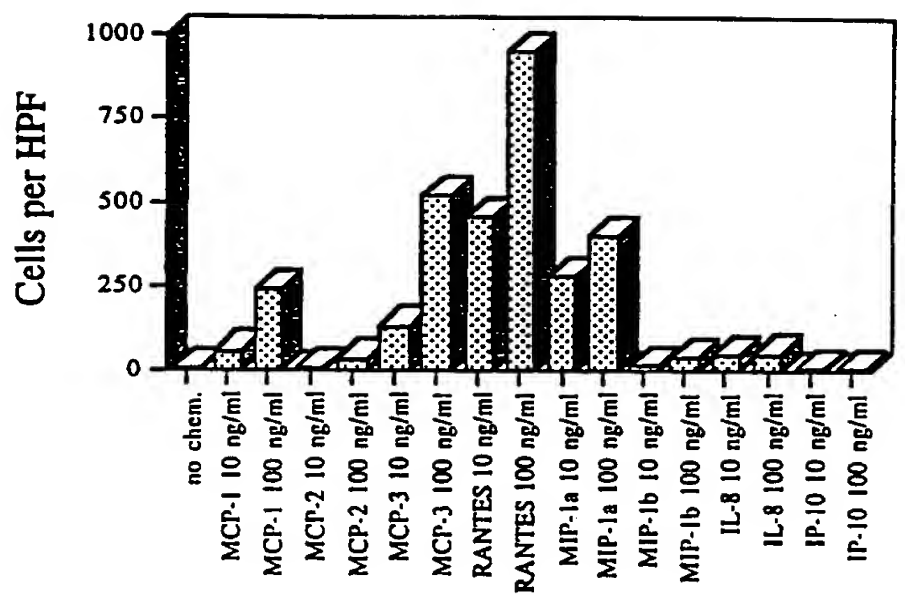


FIG. 4

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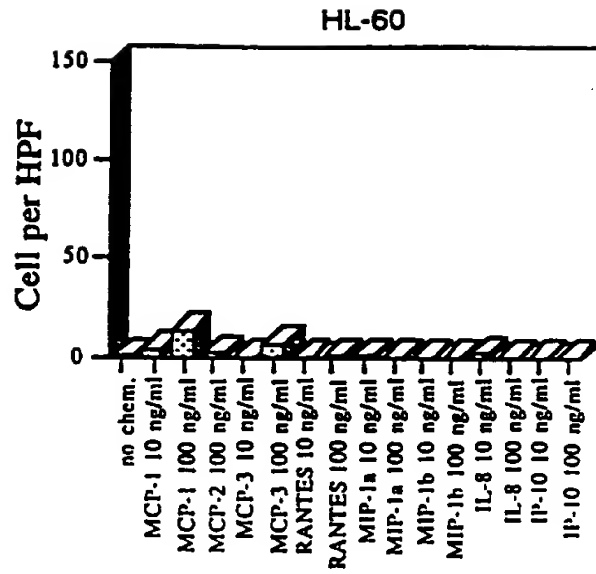


FIG. 5A

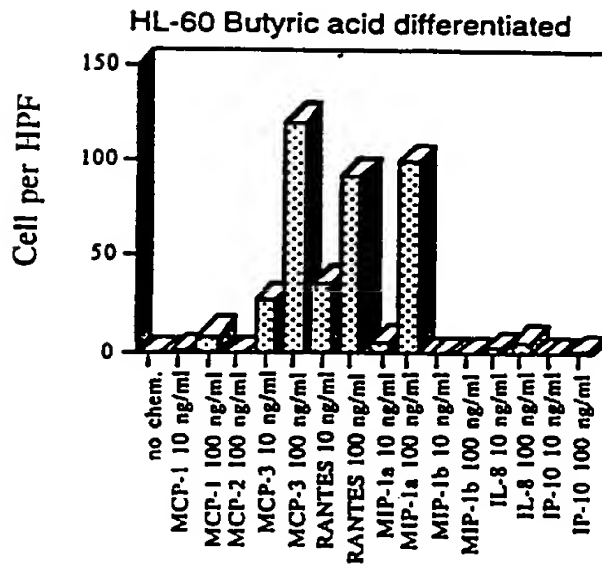


FIG. 5B

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## Expr ssion of Eos L2 on stably transfected L1-2 cells

## Flag staining of different clones

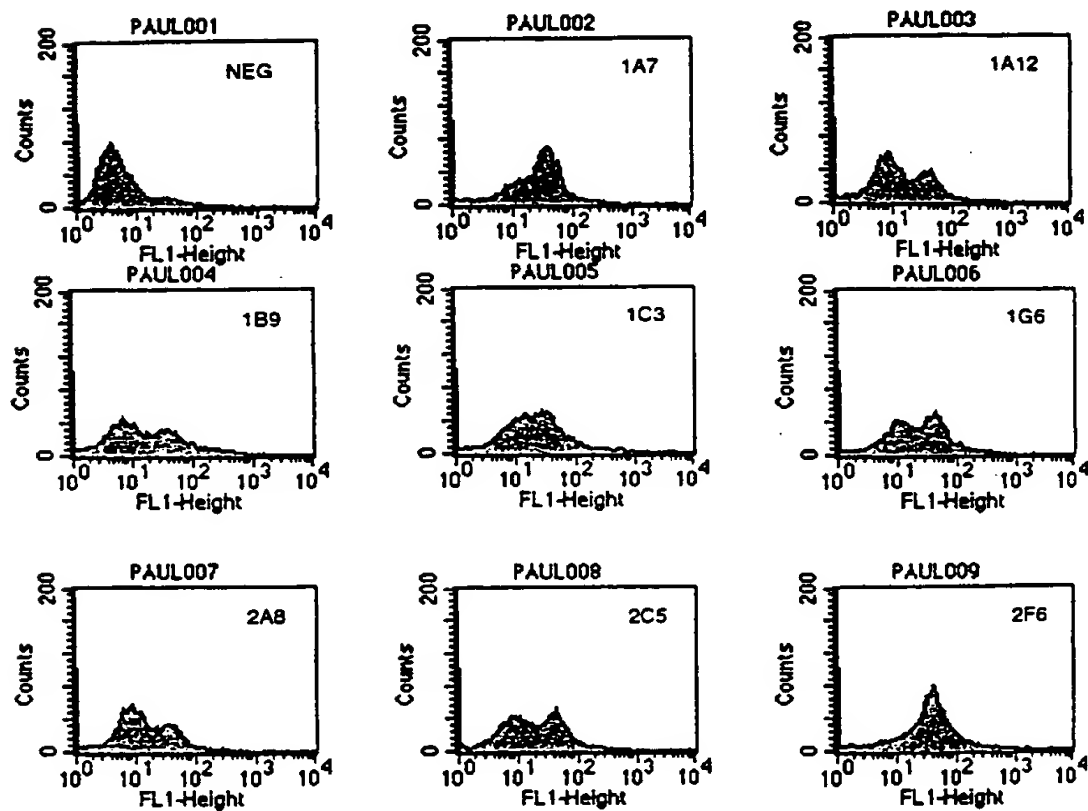


FIG. 6

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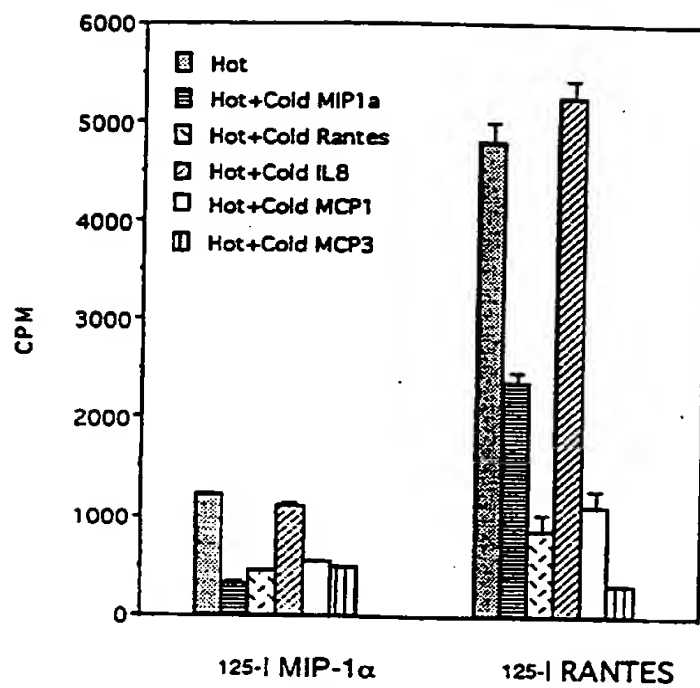


FIG. 7

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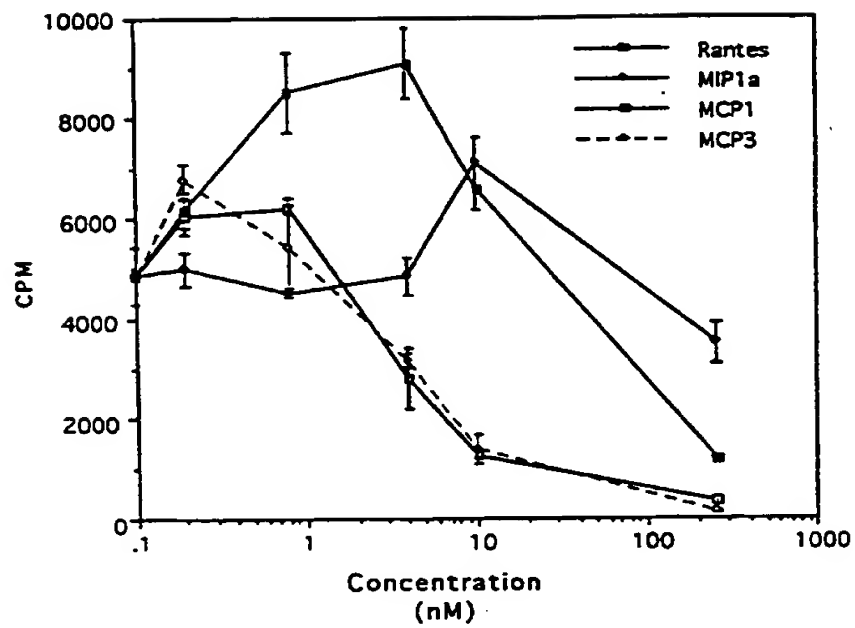


FIG. 8

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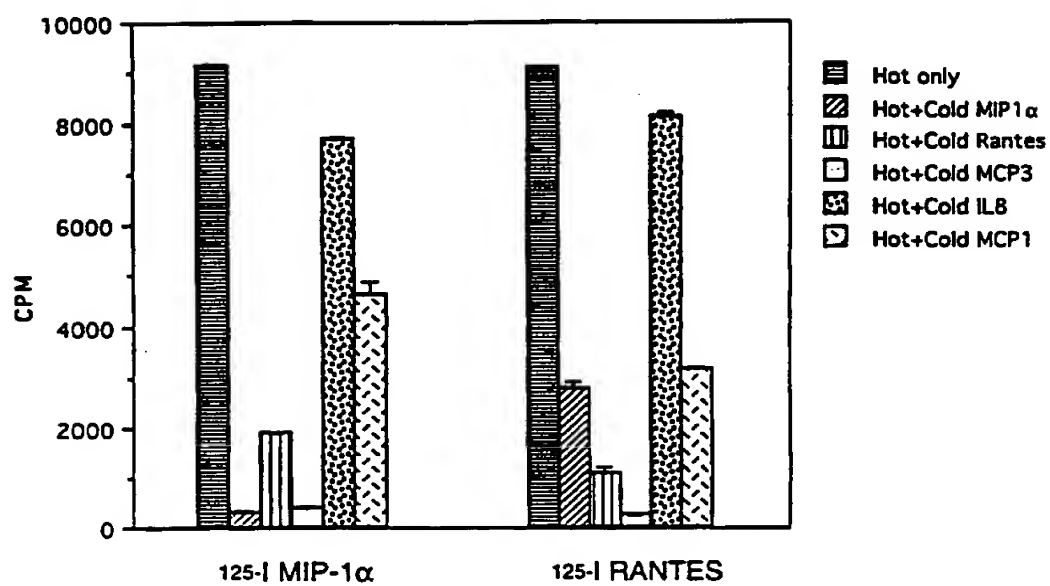


FIG. 9

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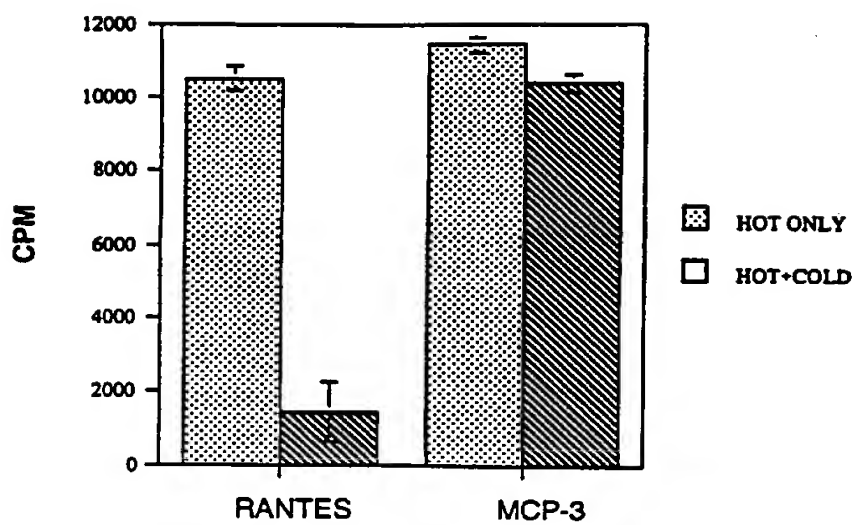


FIG. 10



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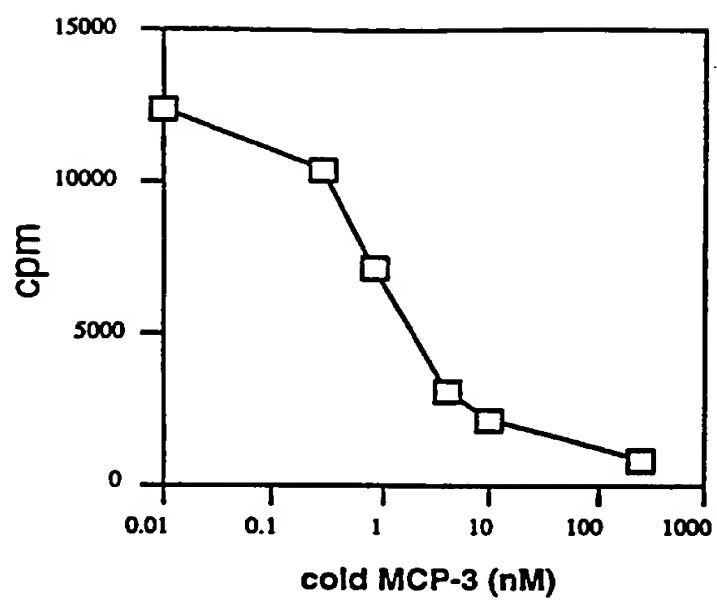


FIG. 11A

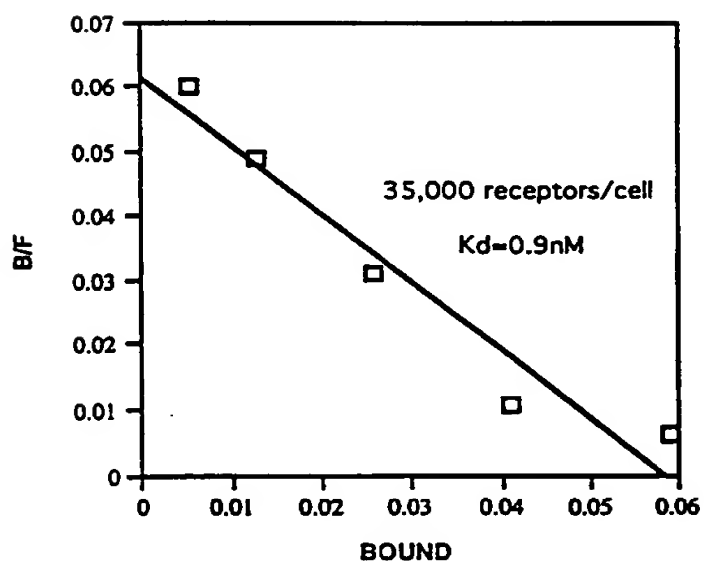


FIG. 11B

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## Human Eosinophils

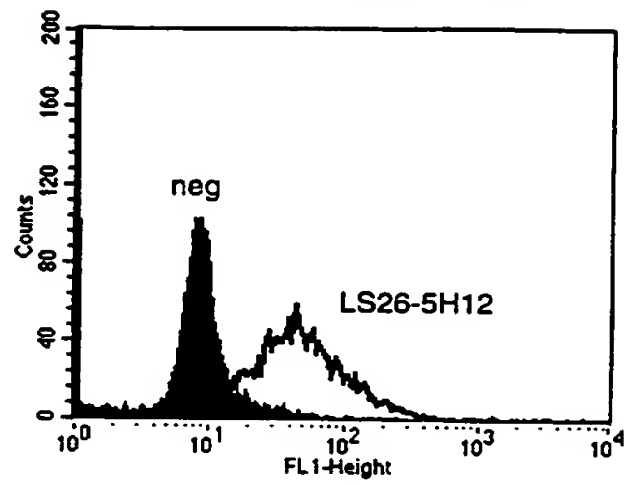


FIG. 12A

## Human Lymphocytes

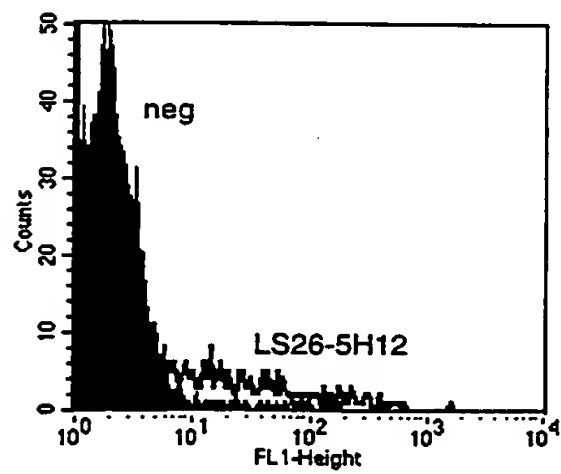


FIG. 12B

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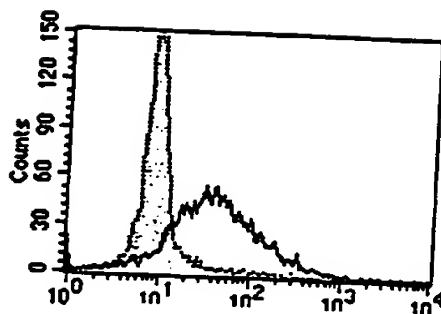


FIG. 13A

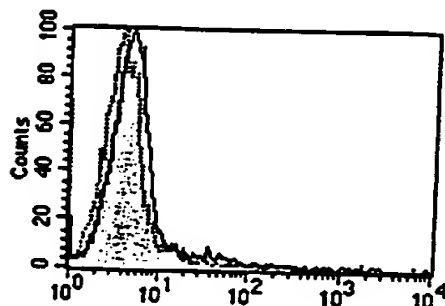


FIG. 13B

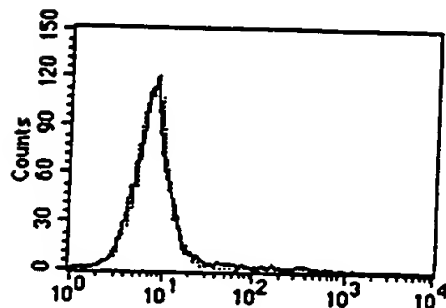


FIG. 13C

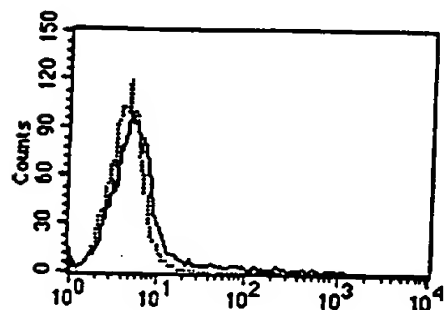


FIG. 13D

Fluorescence intensity →

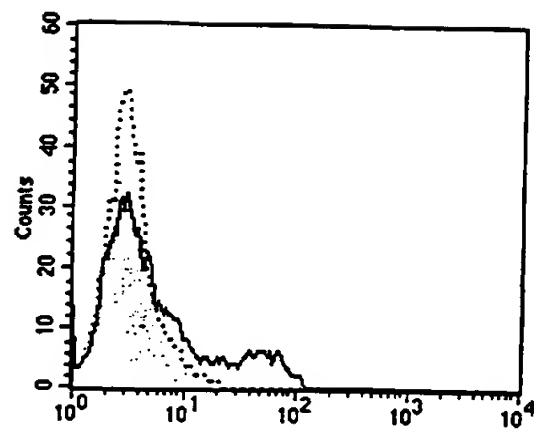


FIG. 14A

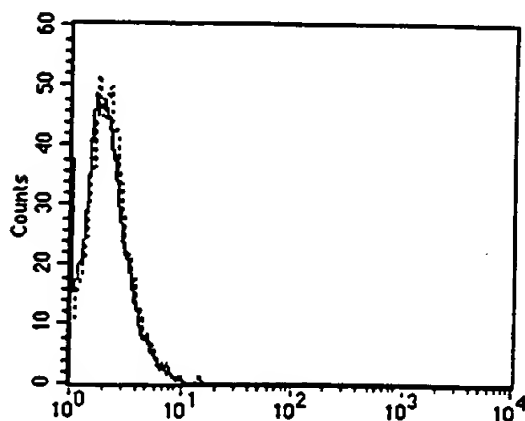


FIG. 14B

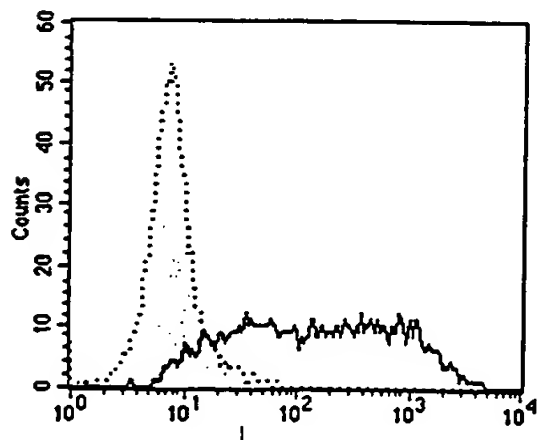


FIG. 14C

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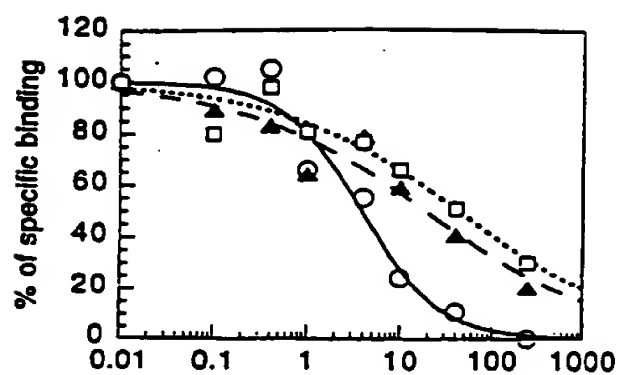


FIG. 15A

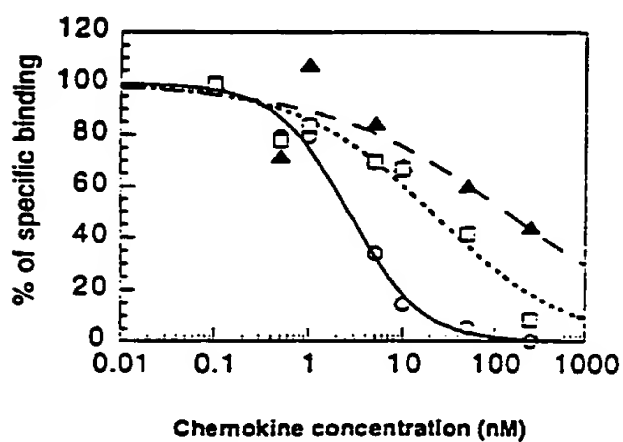


FIG. 15B

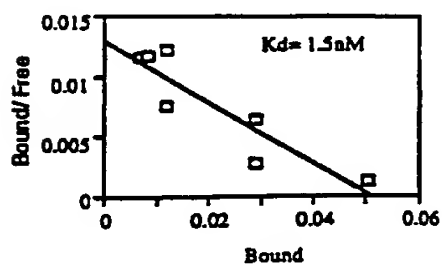


FIG. 15C

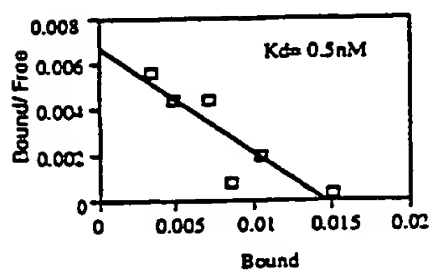


FIG. 15D

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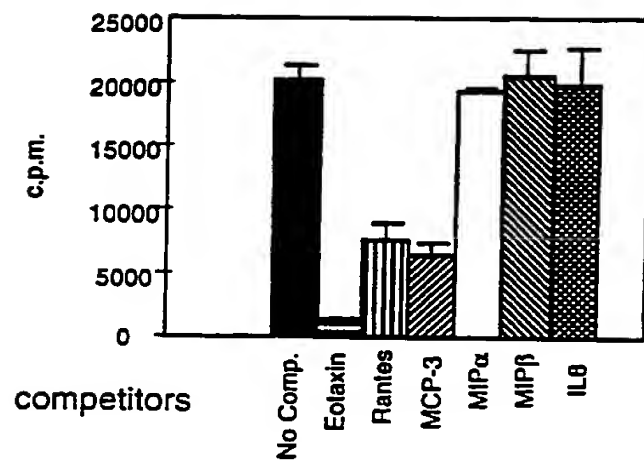


FIG. 16



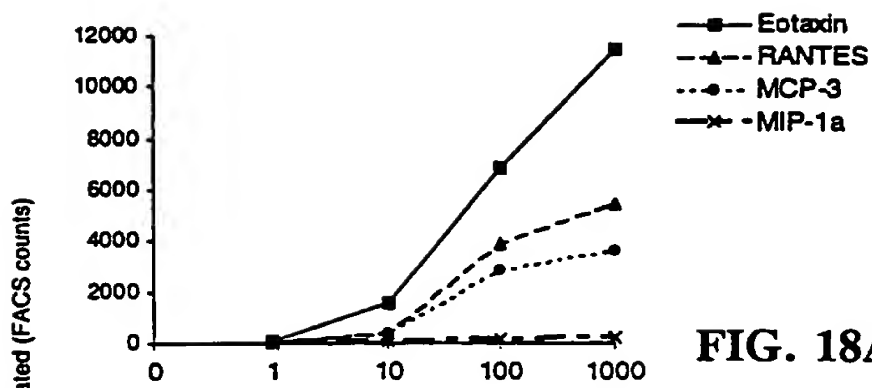


FIG. 18A

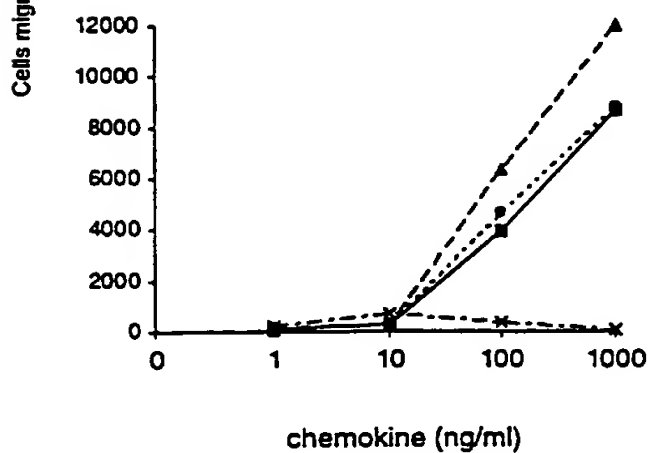


FIG. 18B



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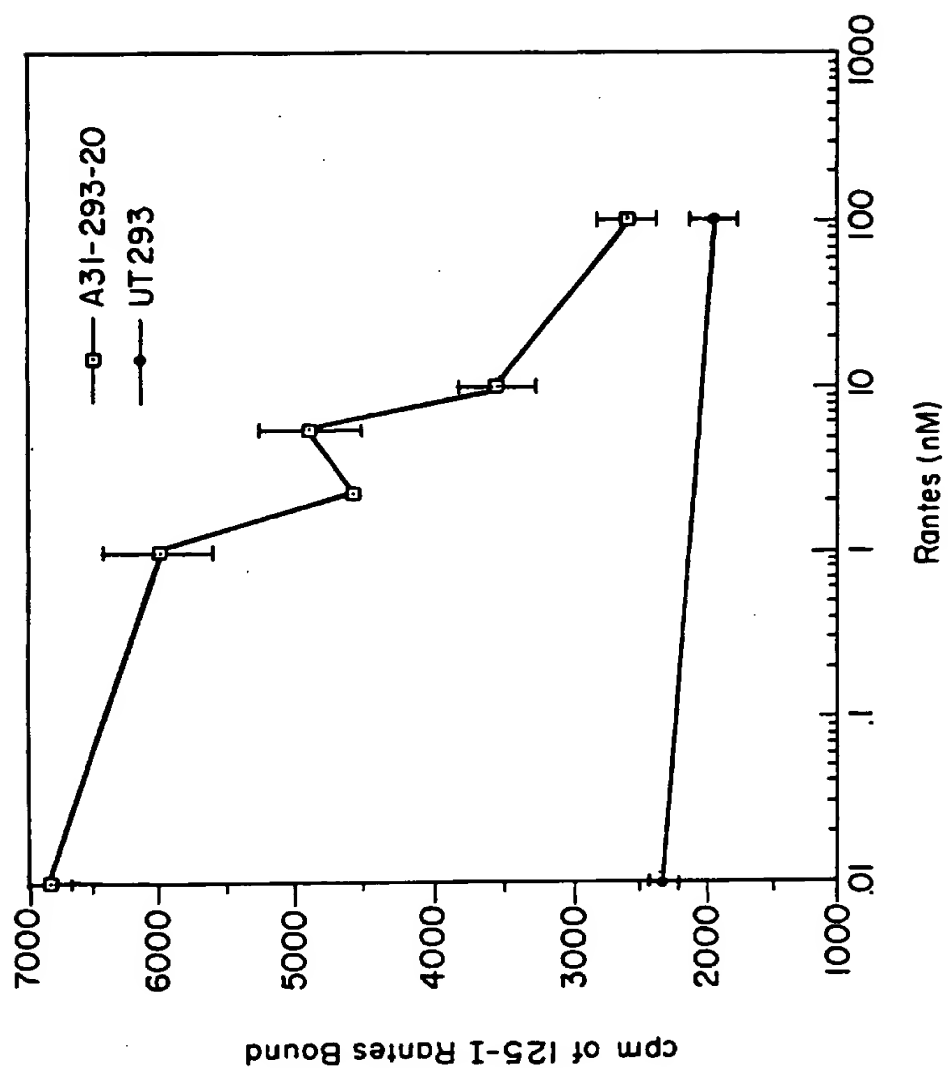


FIG. 19

SUBSTITUTE SHEET (RULE 26)

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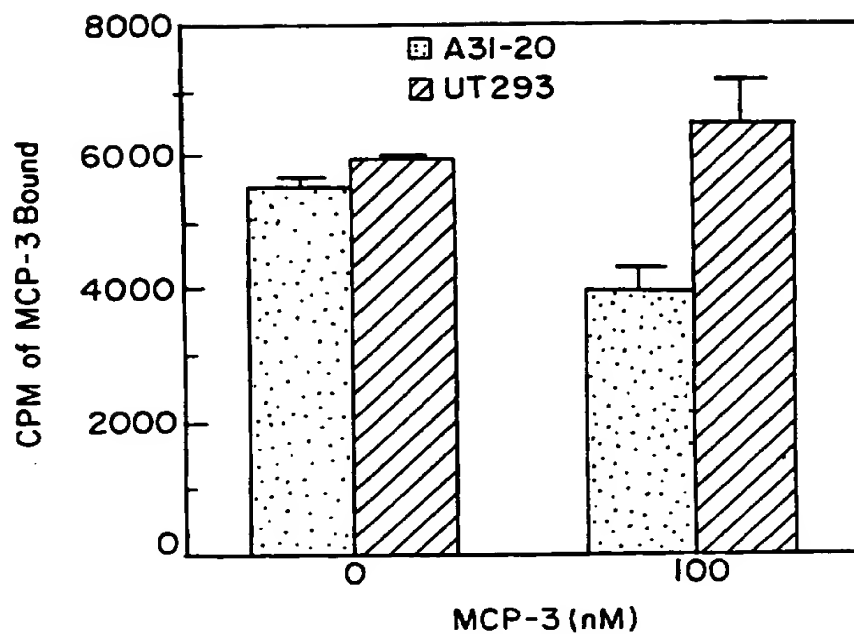


FIG. 20

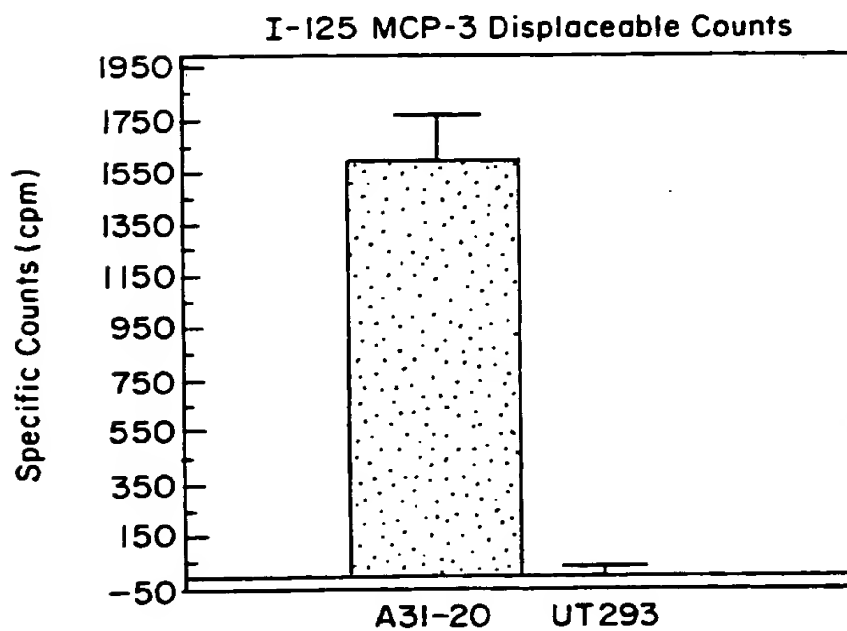


FIG. 21

SUBSTITUTE SHEET (RULE 26)